Microbiology Monographs

Series Editor: Alexander Steinbüchel

Zonglin Lewis Liu *Editor*

Microbial Stress Tolerance for Biofuels

Systems Biology

Springer

Microbiology Monographs
Volume 22

Series Editor: Alexander Steinbüchel
Münster, Germany

For further volumes:
www.springer.com/series/7171

Zonglin Lewis Liu
Editor

Microbial Stress Tolerance for Biofuels

Systems Biology

Springer

Editor
Dr. Zonglin Lewis Liu
National Center for Agricultural Utilization Research
USDA-ARS
1815 N University
Peoria, IL 61604
USA
zlewis.liu@ars.usda.gov

Series Editor
Professor Dr. Alexander Steinbüchel
Institut für Molekulare Mikrobiologie und Biotechnology
Westfälische Wilhelms-Universität
Corrensstr. 3
48149 Münster
Germany
steinbu@uni-muenster.de

ISSN 1862-5576 e-ISSN 1862-5584
ISBN 978-3-642-21466-0 e-ISBN 978-3-642-21467-7
DOI 10.1007/978-3-642-21467-7
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number : 2011937429

Preface

The development of sustainable and renewable biofuels has attracted growing interests with concerns on increased oil demands and a cleaner environment worldwide after decades of attempts since 1950s. Biofuels conversion from renewable biomass including lignocellulosic materials and agricultural residues is considered as the second generation of transportation biofuels. The success of a bio-based economy requests not only the development of an appropriate infrastructure but also the meeting of significant technical challenges for a sustainable industry. The economy of fermentation-based bioprocess including bioethanol production relies extensively on the performance of fermentative microbes. It is vital to develop robust microbial strains for the next generation biocatalysts that are able to function under multiple stress conditions presented in the lignocellulosic biomass-based fermentation systems.

This volume is intended to provide a comprehensive study on microbial stress tolerance using a systems biology approach. It has no means to claim a complete coverage of all important aspects on such a complicated subject by this limited space. The urgency and necessity to address microbial stress tolerance in biofuels applications using comprehensive approaches of systems biology simply cannot be underestimated. In addition to outlining the most advanced knowledge in the respective fields, each chapter provides conclusive remarks and future perspectives intriguing active discussions and proposals. We are humbled to learn that a vast amount of unknown factors exist in detailed life events for microbial stress at the genome level. High levels of integrated interdisciplinary studies are expected to advance basic science on microbial stress tolerance and its applications of successful biofuels productions.

The book consists of two parts of topics around the subject. The first part, comprising the first eight chapters, covers advances and mechanisms of our current understanding on microbial stress tolerance. The second part, comprising the last four chapters, provides approaches and methodology recently developed in related fields with relevant application examples. In the section on advances and mechanisms, genomics aspects are first outlined for yeast tolerance and in situ

detoxification of inhibitory compounds generated during biomass pretreatment. New gene functions, multiple functions of a characterized gene, complex interplay, reprogrammed pathways, and overlapping gene regulatory networks are clearly involved in yeast tolerance at the genome level. A detailed discussion on genetics and regulation of glycogen and trehalose metabolism, important elements for yeast tolerance involved in glycolysis pathways and yeast function, are followed. The sophisticated regulatory system provides insight into yeast tolerance studies not only for nutrition supply but also significant for stressed physiological and toxic status. The next chapter describes molecular mechanisms of programmed cell death as a defensive function against acetic acid, another stress factor concerned about in biofuels fermentation process. Continued pursuing on signal transduction will further advance our understanding on the tolerance to acid stress conditions. The classic yeast *Saccharomyces cerevisiae* is a superb ethanol producing agent, yet it is sensitive to ethanol stress based on varied definitions. A comprehensive discussion on molecular mechanisms of ethanol tolerance by yeast is provided including new data obtained from comparable temporal dynamics of quantitative gene expression analyses. A prototype of molecular mechanisms on ethanol tolerance is presented. From the industrial bioprocessing point of view, the following chapter touches various stress conditions regarding high gravity ethanol fermentation. Bioprocess engineering appears to be a significant component that cannot be overlooked for large scale productions given multiple stress conditions recognized by lignocellulosic biomass conversion. Yeast tolerance has been observed to be closely related to balanced sugar utilizations. An inevitable topic on improving divergent biomass sugar utilization by engineered *S. cerevisiae* is attended in the next chapter. New strategies to improve xylose uptake and utilization by the yeast using synthesized genes and heterologous xylose transporter genes are presented. This advance is expected to lead flourishes of desirable second generation biocatalyst development. These chapters conclude the main coverage on ethanologenic yeast. For bacteria, genomics approaches on tolerance to biomass pretreatment inhibitors by ethanologenic bacterium *Zymomonas mobilis* are summarized. Accurate annotation strategy of the bacterial genome resulted in discoveries of new genes and functions contributing to acetate stress tolerance. Case studies of selected genes involving tolerance and a paradigm of strain development are discussed. In understanding microbial physiology of biofuels production, mechanisms and applications of microbial solvent tolerance are comprehensively addressed in a wide range of bacterial species as well as yeast.

The section on approaches and methodology starts with metabolic engineering using bacterial host for biofuels production in the light of stress tolerance controls. Concerns in developing biofuels producing agents regarding biosynthetic pathways and tolerance mechanisms are discussed with application examples. Such a tolerance is designed against both pretreatment inhibitors and toxic end products. At this end, robust strains encoded by chromosomal integration and free of antibiotic resistant markers are desirable. The next chapter describes basic principles and applications of metabolomics approaches including sample preparation, metabolomic analysis, identification and quantification of metabolites, data mining, and

biological interpretation for gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS) based strategies. A better understanding of metabolomics of microbial stress is expected to benefit optimization of biofuels fermentation processes. The following chapter introduces an automated plasmid-based functional proteomics system. The high throughput platform enables rapid clone and expression of heterologous genes for library screening and improved strain development. The closing chapter presents robust mRNA quantification references that can be used for unified and comparable gene expression data analyses under stress conditions. The fundamental biological process of gene expression raises useful phenotypes in mechanism studies of stress tolerance. Principles and applications as well as critical issues in unification of expression data analysis within and across different platforms of qRT-PCR array and microarray assays are discussed.

We would like to thank all contributing authors for their expertise, efforts, and commitment in this interested project through the entire course of this study, which were essential for the production of this book. We are grateful to Springer for publishing this monograph and special thanks are due to Jutta Lindenborn for her assistance and support. We are also indebted to our families for their unconditional love and support as well as sacrifices of time and leisure during the preparation of this volume.

Peoria, IL, USA — Zonglin Lewis Liu
Münster, Germany — Alexander Steinbüchel

Contents

Genomics of Yeast Tolerance and In Situ Detoxification 1
Z. Lewis Liu

Genetics and Regulation of Glycogen and Trehalose Metabolism in *Saccharomyces cerevisiae* 29
Jean Marie François, Thomas Walther, and Jean Luc Parrou

Molecular Mechanisms of Programmed Cell Death Induced by Acetic Acid in *Saccharomyces cerevisiae* 57
Sergio Giannattasio, Nicoletta Guaragnella, and Ersilia Marra

Molecular Mechanisms of Ethanol Tolerance in *Saccharomyces cerevisiae* 77
Menggen Ma and Z. Lewis Liu

High Gravity Ethanol Fermentations and Yeast Tolerance 117
F.W. Bai and X.Q. Zhao

Improving Biomass Sugar Utilization by Engineered *Saccharomyces cerevisiae* 137
Akinori Matsushika, Z. Lewis Liu, Shigeki Sawayama, and Jaewoong Moon

Genomics on Pretreatment Inhibitor Tolerance of *Zymomonas mobilis* 161
Shihui Yang, Martin Keller, and Steven D. Brown

Mechanisms and Applications of Microbial Solvent Tolerance 177
Mark Taylor, Jean-Baptiste Ramond, Marla Tuffin, Stephanie Burton, Kirsten Eley, and Don Cowan

Control of Stress Tolerance in Bacterial Host Organisms for Bioproduction of Fuels 209
Aindrila Mukhopadhyay, Nathan J. Hillson, and Jay D. Keasling

Metabolomics for Ethanologenic Yeast 239
Ying-Jin Yuan, Ming-Zhu Ding, Jin-Mei Xia, and Jing-Sheng Cheng

Automated Systems of Plasmid-Based Functional Proteomics to Improve Microbes for Biofuel Production 259
Stephen R. Hughes, Tauseef R. Butt, Scott Bartolett, and Steven B. Riedmuller

Unification of Gene Expression Data for Comparable Analyses Under Stress Conditions 279
Z. Lewis Liu

Index 301

Genomics of Yeast Tolerance and In Situ Detoxification

Z. Lewis Liu

Contents

1 Introduction 2
2 Genome Expression Response 3
2.1 Wild Type vs. Tolerant Strains 3
2.2 Induced Expression 4
2.3 Repressed Response 9
3 Aldehyde Reduction Enzymes 10
3.1 New Aldehyde Reductase Genes 10
3.2 New Functions of Characterized Genes 11
3.3 Multiple Functional Enzymes 12
4 Detoxification Pathways 13
4.1 Enhanced Genetic Background 13
4.2 Reprogrammed Regulatory Networks and Redox Balance 14
4.3 Integrated Multiple Gene Interactions 16
5 Genomic Adaptation 17
5.1 YAP Family and YAP1-Regulated Networks 17
5.2 PDR Family and PDR1/3-Involved Cellular Transport Interactions 19
5.3 Protein Modification Interplay Mediated by RPN4, HSF1, and Co-regulators 21
6 Conclusion and Perspectives 22
References 23

Abstract Inhibitory compounds generated by pretreatment of lignocellulose biomass interfere with microbial growth and subsequent fermentation. Remediation of the inhibitors by current physical, chemical, and biological abatement means is economically impractical. Overcoming the inhibitory effects of lignocellulose hydrolysate poses a significant technical challenge for economical cellulosic biofuel production. Development of tolerant ethanologenic yeast has demonstrated

Z.L. Liu (✉)
National Center for Agricultural Utilization Research, USDA-ARS, 1815 N. University Street, Peoria, IL 61604, USA
e-mail: ZLewis.Liu@ars.usda.gov

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_1,

a potential of in situ detoxification for numerous aldehyde inhibitors derived from the biomass pretreatment and conversion. In the last decade, significant progress has been made in understanding mechanisms of yeast tolerance for tolerant strain development. At least, important candidate genes for tolerance have been identified. Enriched genetic backgrounds, enhanced expression, interplay, and global integration of many key genes enable yeast tolerance. Reprogrammed pathways support yeast functions to withstand the inhibitor stress, detoxify the toxic compounds, maintain energy and redox balance, and complete active metabolism for ethanol fermentation. Complex gene interactions and regulatory networks as well as co-regulation are recognized as being involved in yeast adaptation and tolerance. This chapter outlines our current understanding of the yeast tolerance using genome-based approaches.

1 Introduction

Despite the availability of tools and technologies over the last decade for genomics studies, knowledge on real life events at genome level is limited. Genomics, to a degree, is still in its exposure stage. The integration of gene functions, interactions, and regulatory rules at the genome level are far more complex than we can currently imagine. It is humble to learn that a vast amount of the unknown on genomics exists. Yet, we are excited about moving each step forward toward a better understanding of in situ detoxification of lignocellulosic inhibitors involved in cellulosic ethanol conversion by the yeast *Saccharomyces cerevisiae*. This chapter summarizes our current knowledge on mechanisms of the inhibitor detoxification based on molecular studies and genomic-based approaches. Our improved understandings of the in situ detoxification uncover new aspects of global integration and regulation for yeast tolerance and provide insight into phenotype-genotype relationships and strategies for more tolerant strain development in biofuel applications. Two commonly encountered representative inhibitory compounds, 2-furaldehyde (2-furancarbaldehyde; furfural) and 5-(hydroxymethyl)-2-furaldehyde [5-(hydroxythyl)-2-furancarbaldehyde; 5-(hydroxymethyl)-2-furfural; HMF], formed during depolymerization of cellulose and hemicellulose, are the main target inhibitors discussed in this chapter. Background information and comprehensive reviews on the effects of these and other inhibitors related to lignocellulose pretreatment and biomass-to-ethanol conversion are available elsewhere (Larrson et al. 1999; Palmqvist and Hahn-Hägerdal 2000; Klinke et al. 2004; Liu and Blaschek 2010).

Pretreatment of lignocellulosic biomass generates inhibitory compounds that interfere with microbial growth and fermentation and poses a significant challenge for economical cellulosic biofuel production. Remediation of inhibitory compounds by physical and chemical means has been determined to be too expensive for use in practice (Liu et al. 2008a; Liu and Blaschek 2010). A bioabatement method was able to remove aldehyde inhibitors such as furfural; however, additional sugar and carbon source were consumed, and most abatement agents lack fermentation capability

(Nichols et al. 2010). Tolerant ethanologenic yeast strains were found to be able to convert furfural and HMF into less toxic compounds furanmethanol (FM) and furan-2,5-dimethanol (FDM; 2,5-bis-hydroxymethylfuran) respectively while producing ethanol (Liu et al. 2004, 2005, 2008b; Liu 2006; Talebnia and Taherzadeh 2006; Martin et al. 2007). The identification and clarification of FM and FDM as metabolic conversion products of furfural and HMF suggested that the attached aldehyde functional group on the furan ring is responsible for the toxicity but not the furan since numerous other furan compounds are not toxic to yeast (Liu et al. 2004, 2008b; Liu 2006). Historically termed furan inhibitors, furfural and HMF are in fact aldehyde inhibitors. The conversion of the aldehyde functional group into an alcohol form reduces the chemical toxicity. This clarification has led to an attempt to classify the inhibitors by the chemical functional groups to facilitate mechanism studies of in situ detoxification. The current classification of inhibitors contains aldehydes, ketones, phenols, and organic acids commonly associated with lignocellulose hydrolysates and biomass pretreatment procedures (Klinke et al. 2004; Liu and Blaschek 2010). The new classification of the inhibitors has facilitated discoveries of new genes and new functions of known genes. For example, a newly described aldehyde reductase enzyme encoded by *ARI1*, a previously uncharacterized ORF *YGL157W* of *Saccharomyces cerevisiae*, possesses reduction capabilities toward at least 14 aldehydes, including common lignocellulose-derived inhibitors such as furfural, HMF, vanillin, and cinnamaldehyde (Liu and Moon 2009).

2 Genome Expression Response

Gene expression is a fundamental biological process by which phenotype can be recognized in association with genotype. Genome-wide expression responses provide a global view of gene interactions and regulatory network that are important underlining molecular mechanisms of yeast tolerance.

2.1 Wild Type vs. Tolerant Strains

Most gene expression responses in laboratory yeast to environmental stimuli are transient, and a concept of environmental stress response was suggested (Gasch et al. 2000; Gasch and Werner-Washburne 2002). In contrast, industrial ethanologenic yeast gene expression responses appear to be more persistent. Several hundred genes were identified having transcription expression response to furfural and HMF (Liu 2006; Liu and Slininger 2005; 2006; Liu et al. 2009; Ma and Liu 2010; Li and Yuan 2010). These genes are distributed across a wide range of functional categories and pathways including stress-related high-osmolarity glycerol (HOG) pathway, heat shock protein genes, and several important transcription factors (Lin et al. 2009a, b; Li

and Yuan 2010; Ma and Liu 2010). Most studies on yeast response were characterized using a wild-type strain. Recent comparative studies using a tolerant yeast strain, for example, the response to aldehyde inhibitors, provide relevant insight into yeast tolerance (Fig. 1). By comparison of expression profiles and dynamics over time between a wild type and a tolerant strain, tolerance candidate genes can be identified. Such approaches aid studies on mechanisms of tolerance.

2.2 Induced Expression

The inhibitor-induced expression of tolerant yeast consists of only a small portion of genes at the genome level. However, many of these induced genes have multiple functions. Some notable functional categories involve cytoplasm, nucleus, membrane, mitochondrion, cellular protein catabolic process, transport, response to stress, amino acid and derivative metabolic process, hydrolase activity, peptide activity, oxidoreductase activity, protein binding, protein fate, cellular transport, as well as several groups of unknown functions (Tables 1 and 2). At least seven transcription factor genes, *YAP1, YAP5, YAP6, PDR1, PDR3, RPN4,* and *HSF1*, were identified as key regulators for the induced expression response in adaptation to HMF challenge by a tolerant yeast (Song et al. 2009; Ma and Liu 2010). Most of these regulatory genes displayed greater than twofold increase of mRNA abundance after challenges by furfural and HMF. Protein binding motif analysis revealed that each of these transcription factor genes harbors multiple protein binding sites for Pdr3p, Yap1p, Yap5p, Yap6p, Rpn4p, and Hsf1p. For example, DNA binding motifs of Pdr1/3p are present in promoter regions of *PDR3*, *YAP5*, *YAP6*, and *RPN4* (Ma and Liu 2010) (Fig. 2). DNA binding sites of Yap1p and Hsf1p exist in all five transcription factor genes except for *PDR1*, having one Yap1p site, and *PDR3*, two Hsf1p sites. Most transcription factor genes have multiple binding sites for multiple transcription factors. For example, *RPN4* has 13 binding sites of four transcription factors, and *PDR3* has six sites for two. These observations suggest that potential interactions involving multiple transcription factors exist for inhibitor tolerance. High expression of *RPN4* by HMF treatment was suggested to be regulated by Yap1p, Pdr1p, Pdr3p, and Hsf1p based on ChIP-chip assay data, genome expression, and microarray assays of transcription factor mutations (Lee et al. 2002; Harbison et al. 2004; Hahn et al. 2006; Larochelle et al. 2006; Workman et al. 2006; Salin et al. 2008; Ma and Liu 2010). Numerous studies also demonstrated positive feedback of enhanced expression of *RPN4* to its regulators of Yap1p and Pdr1p (Harbison et al. 2004; Haugen et al. 2004; Salin et al. 2008). In addition, DNA binding motif of a transcription factor's own is present in its promoter region, such as *PDR3*, *YAP1,* and *HSF1* (Fig. 2). These suggest a possible self-regulated expression interaction involved in yeast tolerance response as well as co-regulation and interactions of multiple transcription factors under the stressed condition.

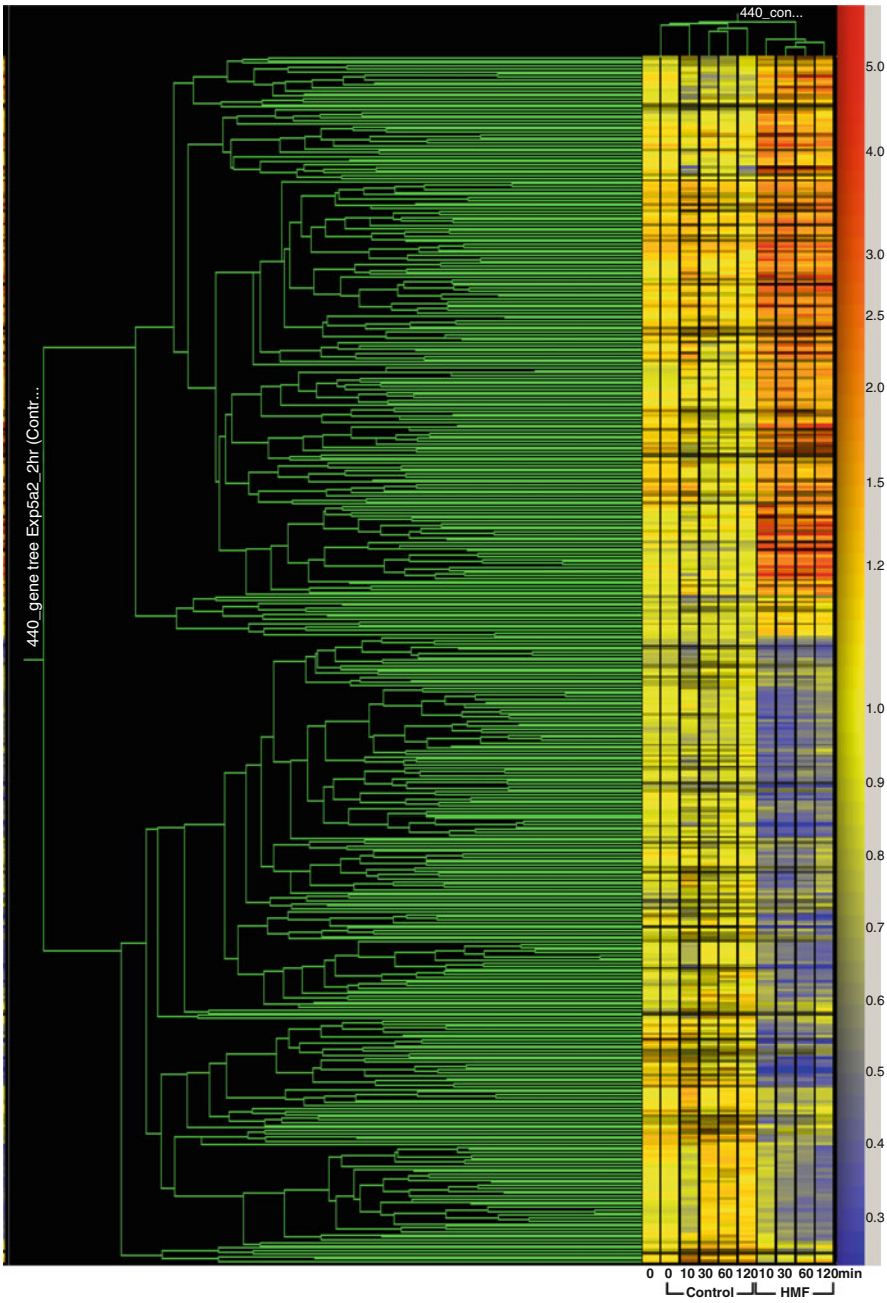

Fig. 1 Comparative interactions of selective genes differentially expressed by HMF challenge over time as examined by genome expression using 70-mer DNA oligo microarray for *Saccharomyces cerevisiae*. *Yellow* indicates an equally expressed mRNA abundance. Varied colors between *yellow* and *red* or *yellow* and *blue* as shown in a *colored bar* at the *far right*, indicate varied quantitative measurements of mRNA expression levels for each gene in a log scale

Table 1 Gene Ontology (GO) categories and terms for significantly induced genes by HMF during the lag phase in *Saccharomyces cerevisiae* (Ma and Liu 2010)

GO ID	GO term	Gene(s)
Cellular component		
GO:0005737	Cytoplasm	***SHP1***[a], ***ATG8***, *YBL107C*, ***HSP26***, ***NPL4***, ***CHA1***, *GPM2*, ***SNQ2***, ***RPN9***, ***SLF1***, ***SSA4***, ***OTU1***, ***RPN12***, ***PYC1***, ***ARI1***, ***YGR111W***, ***ECM29***, ***PUT2***, ***PRE3***, ***MET3***, ***MET14***, ***TPO1***, ***ALT1***, ***PUT1***, ***YAP1***, ***PGA3***, ***ERO1***, ***YNL155W***, ***PRE6***, ***GRE2***, *SGT2*, ***RSB1***, *YOR059C*, ***PDR5***, ***TPO4***, ***PRE10***, ***ALD4***, ***CAR1***
GO:0005634	Nucleus	***SHP1***, ***YBL100W-A***, ***HSP26***, ***RAD16***, ***RPT2***, ***RPN4***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***PRE1***, ***SSA4***, ***MAG1***, ***OTU1***, ***ARI1***, ***YGR111W***, ***ECM29***, *YKR011C*, ***YAP1***, ***YNL155W***, ***GRE2***, *YOR052C*, ***RPT4***
GO:0016020	Membrane	***ATG8***, ***NPL4***, ***SNQ2***, ***PDR15***, ***DDI1***, ***YOR1***, ***TPO1***, ***PGA3***, ***RSB1***, ***PDR5***, ***TPO4***, ***MCH5***, ***PDR12***, *PRM4*
GO:0005575	Cellular component unknown	*IMD1*, *YBR062C*, *YBR255C-A*, *YDR034W-B*, *YER137C*, *YGR035C*, ***YHR138C***, *YLL056C*, *ICT1*, *OYE3*
GO:0005886	Plasma membrane	***SNQ2***, ***DDI1***, ***YOR1***, ***TPO1***, ***PGA3***, ***RSB1***, ***PDR5***, ***TPO4***, ***MCH5***, ***PDR12***
GO:0005739	Mitochondrion	***CHA1***, ***SNQ2***, ***PUT2***, ***MET3***, ***ALT1***, ***PUT1***, ***PRE6***, ***PDR5***, ***ALD4***
GO:0005783	Endoplasmic reticulum	***NPL4***, ***PGA3***, ***ERO1***, ***RSB1***
GO:0005773	Vacuole	***ATG8***, ***TPO1***, ***TPO4***
GO:0005624	Membrane fraction	***SNQ2***, ***YOR1***
GO:0005933	Cellular bud	***TPO1***
GO:0005618	Cell wall	*TIR4*
GO:0012505	Endomembrane system	***NPL4***
GO:0030427	Cite of polarized growth	***CAR1***
Other	Other	***PRE7***, *ADH7*, ***RPT3***, ***PUP3***
Biological process		
GO:0008150	Biological process unknown	*IMD1*, *YBL107C*, *YBR062C*, *YBR255C-A*, *GPM2*, *YDR034W-B*, *YER137C*, ***ARI1***, *YGR035C*, *YKR011C*, *YLL056C*, ***YNL155W***, *TIR4*, *YOR052C*, *YOR059C*, *PRM4*, *OYE3*
GO:0044257	Cellular protein catabolic process	***PRE7***, ***SHP1***, ***RAD16***, ***NPL4***, ***RPT2***, ***RPT3***, ***RPN9***, ***PRE1***, ***PUP3***, ***DDI1***, ***RPN12***, ***PRE3***, ***PRE6***, ***RPT4***, ***PRE10***
GO:0006810	Transport	***ATG8***, ***PDR15***, ***SSA4***, ***DDI1***, ***YOR1***, ***TPO1***, ***PGA3***, ***RSB1***, ***PDR5***, ***TPO4***, ***MCH5***, ***PDR12***
GO:0006950	Response to stress	***ATG8***, ***HSP26***, ***RAD16***, ***RPN4***, ***SNQ2***, ***PRE1***, ***SSA4***, ***MAG1***, ***PRE3***, ***YAP1***, *SGT2*
GO:0042221	Response to chemical stimulus	***RPN4***, ***SNQ2***, ***PDR15***, ***YOR1***, ***MET14***, ***YAP1***, ***PDR5***
GO:0006519	Cellular amino acid and derivative metabolic process	***CHA1***, ***PUT2***, ***MET3***, ***MET14***, ***ALT1***, ***PUT1***, ***CAR1***
GO:0032196	Transposition	***YBL100W-A***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s)
GO:0006457	Protein folding	***HSP26***, ***SSA4***, ***ERO1***
GO:0006350	Transcription	***RPN4***, ***OTU1***, ***YAP1***
GO:0006464	Protein modification process	***RAD16***, ***OTU1***, ***ERO1***
GO:0030435	Sporulation resulting in formation of a cellular spore	***SHP1***, ***PRE1***, ***PRE3***
GO:0006259	DNA metabolic process	***RAD16***, ***RPN4***, ***MAG1***
GO:0016044	Membrane organization	***ATG8***, ***YHR138C***, ***RSB1***
GO:0007033	Vacuole organization	***ATG8***, ***YHR138C***
GO:0044262	Cellular carbohydrate metabolic process	***SHP1***, ***PYC1***
GO:0044255	Cellular lipid metabolic process	*ICT1*, ***GRE2***
GO:0006766	Vitamin metabolic process	***PYC1***, ***ALD4***
GO:0046483	Heterocycle metabolic process	***PUT2***, ***PUT1***
GO:0051186	Cofactor metabolic process	***PYC1***, ***ALD4***
GO:0016192	Vesicle-mediated transport	***ATG8***, ***DDI1***
GO:0051276	Chromosome organization	***RAD16***
GO:0016070	RNA metabolic process	***YAP1***
GO:0006412	Translation	***SLF1***
GO:0006091	Generation of precursor metabolites and energy	***SHP1***
GO:0070271	Protein complex biogenesis	***RPN9***
GO:0007049	Cell cycle	***RPN4***
GO:0019725	Cellular homeostasis	***SLF1***
Other	Other	*ADH7*, ***YGR111W***, ***ECM29***
Molecular function		
GO:0016787	Hydrolase activity	***PRE7***, ***RAD16***, ***RPT2***, ***SNQ2***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***RPT3***, ***PDR15***, ***PRE1***, ***PUP3***, ***SSA4***, ***MAG1***, ***OTU1***, ***RPN12***, ***YOR1***, ***PRE3***, ***PRE6***, ***RSB1***, ***PDR5***, ***RPT4***, ***PRE10***, ***PDR12***, ***CAR1***
GO:0003674	Molecular function unknown	*IMD1*, ***ATG8***, *YBL107C*, *YBR062C*, ***NPL4***, *YBR255C-A*, *GPM2*, *YDR034W-B*, *YER137C*, *YGR035C*, ***YGR111W***, *YKR011C*, *YLL056C*, ***PGA3***, ***YNL155W***, *SGT2*, *TIR4*, *YOR052C*, *YOR059C*, *PRM4*
GO:0008233	Peptidase activity	***PRE7***, ***RPT2***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***RPT3***, ***PRE1***, ***PUP3***, ***OTU1***, ***RPN12***, ***PRE3***, ***PRE6***, ***RPT4***, ***PRE10***
GO:0005215	Transporter activity	***SNQ2***, ***PDR15***, ***YOR1***, ***TPO1***, ***RSB1***, ***PDR5***, ***TPO4***, ***MCH5***, ***PDR12***
GO:0016491	Oxidoreductase activity	*ADH7*, ***ARI1***, ***PUT2***, ***PUT1***, ***ERO1***, ***GRE2***, ***ALD4***, *OYE3*
GO:0005515	Protein binding	***YBL100W-A***, ***HSP26***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***SSA4***, ***DDI1***, ***ECM29***

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s)
GO:0016740	Transferase activity	***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***MET3***, ***MET14***, ***ALT1***, *ICT1*
GO:0003723	RNA binding	***YBL100W-A***, ***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***SLF1***
GO:0016779	Nucleotidyltransferase activity	***YDR210W-B***, ***YDR316W-B***, ***YDR365W-B***, ***MET3***
GO:0003677	DNA binding	***RAD16***, ***RPN4***, ***YAP1***
GO:0016874	Ligase activity	***RAD16***, ***PYC1***
GO:0030528	Transcription regulator activity	***RPN4***, ***YAP1***
GO:0030234	Enzyme regulator activity	***SHP1***, ***YHR138C***
GO:0016829	Lyase activity	***CHA1***
GO:0005198	Structural molecule activity	***RPN9***
GO:0016853	Isomerase activity	*GPM2*

[a]Genes in bold indicate that their encoding proteins or enzymes are involved in more than one function

Table 2 Protein functional categories for significantly induced genes by HMF during the lag phase in *Saccharomyces cerevisiae* (Ma and Liu 2010)

MIPS ID	Functionary category	p-value	Entries
01 Metabolism			
01.01.03.03.02	Degradation of proline	7.82E-04	PUT2, **PUT1**[a]
01.01.03.05.02	Degradation of arginine	3.94E-04	**PUT1**, CAR1
01.02.03.01	Sulfate assimilation	3.54E-03	MET3, MET14
14 Protein fate (folding, modification, destination)			
14.07.11	Protein processing (proteolytic)	4.05E-09	**PRE7**, **ATG8**, **RPT2**, **RPT3**, **PRE1**, **PUP3**, **RPN12**, **PRE3**, **PRE6**, **RPT4**, **PRE10**
14.13	Protein/peptide degradation	3.97E-11	**PRE7**, SHP1, **ATG8**, NPL4, **RPT2**, RPN4, **RPT3**, RPN9, **PRE1**, **PUP3**, DDI1, OTU1, **RPN12**, ECM29, YHR138c, **PRE3**, **PRE6**, **RPT4**, **PRE10**
16 Protein with binding function or cofactor requirement (structural or catalytic)			
16.19.03	ATP binding	1.52E-03	**RPT2**, **SNQ2**, **RPT3**, **PDR15**, **YOR1**, **PDR5**, **RPT4**, **PDR12**
20 Cellular transport, transport facilities, and transport routes			
20.01.27	Drug/toxin transport	4.70E-06	**SNQ2**, **YOR1**, TPO1, **PDR5**, TPO4, **PDR12**
20.03.22	Transport ATPases	3.68E-04	**SNQ2**, **YOR1**, RSB1, **PDR5**, **PDR12**
20.03.25	ABC transporters	1.44E-05	**SNQ2**, **PDR15**, **YOR1**, **PDR5**, **PDR12**
32 Cell rescue, defense, and virulence			
32.05.01.03	Chemical agent resistance	1.73E-05	**SNQ2**, MAG1, **YOR1**, YAP1, **PDR5**

[a]Proteins in bold indicate functions involved in more than one category

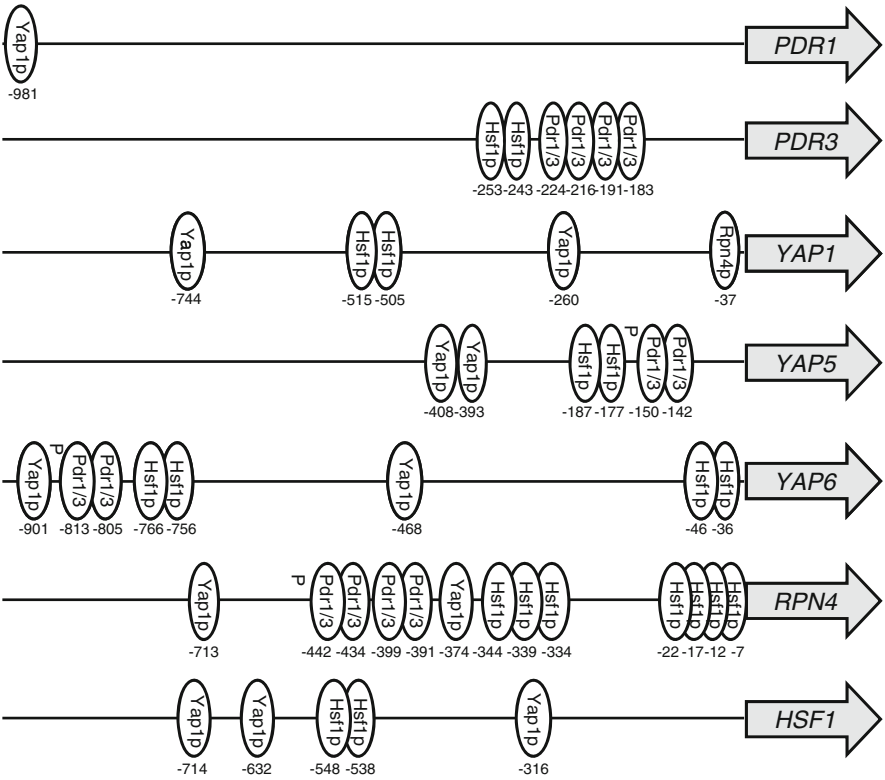

Fig. 2 DNA binding sites in the promoter regions from −1,000 to −1 for seven selective transcription factor genes *YAP1*, *YAP5*, *YAP6*, *PDR1*, *PDR3*, *RPN4,* and *HSF1* of *Saccharomyces cerevisiae*, showing overlapping and multiple binding sites that indicate gene co-regulation roles of key transcription factor genes (Ma and Liu 2010)

2.3 Repressed Response

Most differentially expressed genes show repressed response to inhibitor challenges regardless of strain and the treatment methods used. The difference lies in that repressed genes in certain categories are able to recover over time while others remain repressed as demonstrated by comparative transcription dynamic analyses (Liu et al. 2009; Ma and Liu 2010). The importance of repressed genes is often neglected in contrast to overwhelmingly emphasized attention to the induced genes. In fact, many of these "overlooked" genes play necessary roles in yeast adaptation as they are able to recover and function under stress. As mentioned, the lack of such functional genes can result in nonviable biological processes, including ethanol fermentation. Under certain conditions, down-regulated expression could be an efficient means of energy utilization for economic pathway development (Ma and Liu 2010). The repressed genes are mainly involved in the functional categories of

ribosome biogenesis, amino acid and derivative metabolic process, RNA synthesis, RNA metabolic process, transport, transcriptional and translation controls, mitochondrial, and others (Ma and Liu 2010; Li and Yuan 2010). For many repressed genes, at least five important regulatory genes, including *ARG80, ARG81, GCN4, RAP1,* and *FHL1*, were found to be involved in the significantly down-regulated expression. For example, *ARG1*, *ARG3*, *ARG4*, *ARG5, ARG6*, *ARG7,* and *ARG8* involved in arginine biosynthesis and repressed by HMF were regulated by the transcription factor genes *ARG80* and *ARG81* as well as *GCN4* (De Rijcke et al. 1992; Natarajan et al. 2001; Ma and Liu 2010). In addition to regulation of arginine biosynthesis, *GCN4* regulates expression of many other genes related to amino acid biosynthesis, such as a number of genes involved in biosynthesis of histidine, leucine, and lysine (Natarajan et al. 2001; Ma and Liu 2010). Among the many genes repressed by HMF, a large number of genes are involved in ribosome biogenesis and protein translation processes, which were predicted to be regulated by transcription factor genes *RAP1* and *FHL1*.

3 Aldehyde Reduction Enzymes

Classification and clarification of furfural and HMF as aldehyde inhibitors allowed identification of aldehyde reduction enzymes. The aldehyde reduction function was found not only in numerous previously reported genes but also for uncharacterized ORFs. It appears as a common functional category for many oxidoreductase genes in yeast.

3.1 New Aldehyde Reductase Genes

A novel gene encoding NADPH-dependent aldehyde reductase, *ARI1*, was characterized recently (Liu and Moon 2009; Bowman et al. 2010; Saccharomyces Genome Database http://www.yeastgenome.org/). The product of *ARI1* is the first purified yeast protein reported as an aldehyde reductase involved in the detoxification of inhibitors of lignocellulose hydrolysates. As mentioned, it has reduction activities toward at least 14 aldehydes, including those frequently identified during biomass pretreatment procedures. The optimum performance temperature of the enzyme is 25°C at pH 7.0. The protein of *ARI1* has an approximate molecular mass of 38 kDa and is a member of the "intermediate" subclass of the SDR (short-chain dehydrogenase/reductase) superfamily with the following typical characteristics: The conserved catalytic site lies at Tyr_{169}-X-X-X-Lys_{173}; an indispensable reduction catalytic tetrad, at Asn_{106}, Ser_{131}, Tyr_{169}, and Lys_{173}; and an approved cofactor binding motif, at Gly_{11}-X-X-Gly_{14}-X-X-Ala_{17} near the N-terminus. The function of the gene was annotated by conserved functional sequence motifs, gene expression, protein expression, and partially purified protein assays. This newly described gene

possibly represents a group of uncharacterized multiple functional genes such as other potential candidates *YKL071W*, Y62, Y76, Y81, and Y82 (Heer et al. 2009; Liu and Blaschek 2010; Liu and Moon 2010).

3.2 New Functions of Characterized Genes

Understanding the importance of detoxification of the aldehyde functional group has allowed recognition of numerous enzymes possessing aldehyde reductase activity that contribute to the detoxification of the aldehyde inhibitors associated with lignocellulose pretreatment, such as furfural, HMF, cinnamaldehyde, and vanillins. Several previously reported genes were found to posses new functions of aldehyde reduction. For example, yeast clones overexpressing *ADH6* and *ADH7* displayed high reduction capabilities toward furfural and HMF (Table 3). Although they were characterized as alcohol dehydrogenases, the kinetic study of ADH6 and ADH7 showed that their reductive reactions were 50- to 100-fold more efficient than the corresponding oxidations (Larroy et al. 2002a, b, 2003). It is possible that ADH6 or ADH7 act as an aldehyde reductase rather than as an alcohol dehydrogenase as their major metabolic function. Cell protein extracts of mutated *ADH1*-containing yeast

Table 3 Genes encoding enzymes possessing aldehyde reductase activities examined by the enzyme specific activity using whole cell protein extract or partially purified proteins exampled by furfural and HMF for ethanologenic strains of *Saccharomyces cerevisiae*

Gene	Enzyme Commission number	Cofactor	Substrate	Enzyme specific activity (mU/mg protein)	Reference
ADH6	1.1.1.2	NADPH	Furfural	98–4,000	Petersson et al. 2006; Liu et al. 2008b; Almedia et al., 2009
			HMF	79–4,000	
		NADH	Furfural	62–210	Petersson et al. 2006; Liu et al. 2008b
			HMF	ns[a]	
ADH7	1.1.1.2	NADH	Furfural	86	Liu et al. 2008b
			HMF	158	
ALD4	1.2.1.5	NADH	Furfural	67	Liu et al. 2008b
			HMF	93	
GRE3	1.1.1.- 1.1.1.21	NADH	Furfural	115	Liu et al. 2008b
			HMF	157	
ADH1	1.1.1.1 1.1.1.190	NADH	Furfural	~3,900	Almeida et al. 2008
			HMF	~3,800	
ARI1	1.1.1.-	NADPH	Furfural	4,290	Liu and Moon 2009
			HMF	580	
GRE2	1.1.1.283	NADH	Furfural	540	Moon and Liu 2011
			HMF	50	
Y62	1.1.1.-	NADH	Furfural	349	Liu and Blaschek 2010
Y76	1.1.1.-	NADH	Furfural	353	Liu and Blaschek 2010

[a]Not significant

strains also showed significant aldehyde reductase activities (Almeida et al. 2008; Laadan et al. 2008). ALD4 is a major mitochondrial aldehyde dehydrogenase that is required for growth on ethanol and the conversion of acetaldehyde to acetate using $NADP^+$ or NAD^+ as a coenzyme. This enzyme is also able to reduce HMF and furfural utilizing NADH as a cofactor. Aldehyde dehydrogenase is known to play an important role in yeast acetaldehyde metabolism. Thus, aldehyde dehydrogenase could be another potential candidate gene for detoxification of aldehyde inhibitors. Similarly, aldo-keto reductase and methylglyoxal-related reductases GRE3 and GRE2 showed aldehyde reduction activities (Liu et al. 2008b; Liu and Moon 2009; Moon and Liu 2011). Xylose reductase from *Pichia stipitis* and expressed in *S. cerevisiae* also possessed reduction activities toward furfural and HMF (Almeida et al. 2008). In addition to their significant involvement under stress conditions, these enzymes appeared to be important candidates facilitating inhibitor reduction. The *GRE3* has been deleted in an effort to reduce xylitol byproduct production to improve xylose utilization efficiency of yeast (Träff et al. 2001; Kuyper et al. 2005). Considering the significant interaction between inhibitor tolerance and efficient pentose utilization, it is worthwhile to clarify the roles and interplay among the important candidate gene groups for balanced metabolic function in biofuel conversions by yeasts.

3.3 Multiple Functional Enzymes

Detoxification of the aldehyde inhibitors is accomplished by reduction activities coupled with cofactors NADH and/or NADPH in multiple aldehyde reductase enzymes (Morimoto and Murakami 1967; Nemirovskii et al. 1989; Villa et al. 1992; Wahlbom and Hahn-Hägerdal 2002; Liu et al. 2004, 2008b; Nilsson et al. 2005; Liu 2006; Petersson et al. 2006; Liu and Moon 2009; Almeida et al. 2008; Liu and Blaschek 2010) (Fig. 3). Most in vitro enzyme assays for reduction of furfural and HMF were evaluated using whole cell protein extracts; however, a few examples using partially purified proteins are available (Liu and Moon 2009; Moon and Liu 2010). Some notable enzymes, such as alcohol dehydrogenase *ADH7*, *ADH6,* and *ADH1,* aldehyde dehydrogenase *ALD4*, and methylglyoxal reductase *GRE2* and *GRE3*, have been demonstrated to possess efficient aldehyde reduction activities (Table 3). Comparative proteomic analysis of an industrial yeast strain suggested Adh5p and Adh1p as the catalytic agents for furfural reduction (Lin et al. 2009a). Protein extracts from individual gene clones often show distinct cofactor preference. However, whole cell protein extracts from a tolerant ethanologenic yeast display strong aldehyde reduction activities with either NADH or NADPH and do not appear to have a strong cofactor preference (Liu et al. 2008b). A single gene deletion of the related reductase does not appear to significantly affect the detoxification capacity in yeast. It is likely that yeasts are able to respond at multiple levels in biotransformation of the aldehyde inhibitors. Transcriptome analysis indicated that many reductase genes were immediately

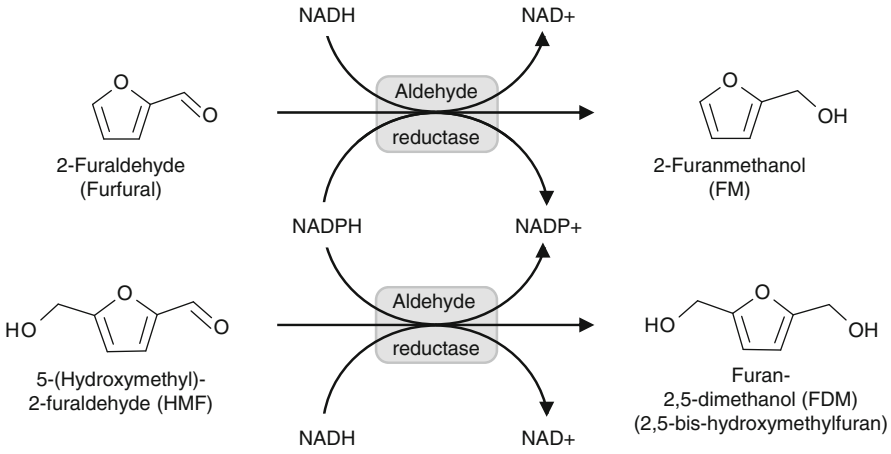

Fig. 3 Conversion pathways of 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (HMF) into 2-furanmethanol (FM) and furan-2,5-dimethanol (FDM) coupled with NADH and/or NADPH and catalyzed by multiple reductases (Liu 2011)

induced by the toxic treatment, such as *ADH7*, *ARI1*, *GRE2,* and *ALD4* (Ma and Liu 2010). Among these, *ADH7* can have a greater than 30- to 80-fold increase in transcript abundance after the HMF addition at 10 min and 1 h, respectively. As demonstrated by ^{13}C-labeled metabolic flux and transcription study, *ADH7* and ORF *YKL071W*, and possibly four other reductases, are associated with the yeast resistance to the furfural challenge (Heer et al. 2009).

4 Detoxification Pathways

Yeast detoxification pathways for aldehyde inhibitors are recently established by utilizing tolerant ethanologenic yeast that is able to in situ detoxify the inhibitors. In addition to the specific steps for the aldehyde reduction, global gene response to the inhibitory compounds, specifically for that cofactor regeneration, and glycolysis-related genes play important roles in integrated gene interactions for tolerance.

4.1 Enhanced Genetic Background

A tolerant yeast is able to withstand challenges of high levels of furfural–HMF inhibitor complex and produce normal yields of ethanol while the parental strain fails to establish a viable culture under the same conditions (Liu et al. 2009). It is clear that the tolerant yeast possesses different genetic mechanisms for in situ detoxification of the toxic compounds that enable active metabolism for ethanol

production. Characterization of gene expression dynamics of the tolerant yeast strain suggested that the tolerant yeast appeared to have an inheritable genetic makeup that is distinct from its parental strain. At least 16 gene transcripts involved in glucose metabolism displayed significantly greater abundance in the inhibitor-tolerant yeast compared with its parental strain even without the inhibitor treatment (Liu et al. 2009). Many of these are key genes involved in the glucose metabolic process, NAD(P)H metabolic and regeneration, and transferase activities such as *HXK1, HXK2, GLK1, TDH1, TDH3, LAT1, PDC6, ADH4, ALD2, ALD4, ZWF1, SOL3, RBK1, TAL1, NQL1,* and *PRS2* (Table 1). Some of these key genes displayed as high as 4 to 6-fold increased abundance, for example, the hexokinase encoding genes *HXK1* and *HXK2,* glyceraldehyde-3-phosphate dehydrogenase gene *TDH1*, dihydrolipoamide acetyltransferase component (E2) of the pyruvate dehydrogenase complex gene *LAT1,* major mitochondrial aldehyde dehydrogenase gene *ALD4*, and *TAL1* that encodes transaldolase.

4.2 Reprogrammed Regulatory Networks and Redox Balance

Glycolysis and pentose phosphate pathway are closely related pathways in yeast glucose metabolism. This close relationship is of such importance that the two pathways cannot be viewed separately in discussing yeast tolerance and detoxification of the lignocellulose inhibitors. Under the challenge of furfural–HMF complex, yeast is unable to grow, and most genes involved in these pathways are severely repressed. A tolerant yeast strain, on the other hand, demonstrated different expression dynamics and completed ethanol fermentation. Under inhibitor stress, high levels of expression by *HXK1, HXK2,* and *GLK1* appeared to secure the initiation stage of phosphorylation of glucose by these enzyme encoding genes (Liu et al. 2009). Then, the significantly induced expression of *ZWF1, SOL3, GND1*, and *GND2*, as well as the repression of glycolytic enzyme phosphoglucose isomerase, apparently drive the glucose metabolism toward pentose phosphate pathway. Gene deletion mutations of *ZWF1* and *GND1* are highly sensitive to furfural and HMF (Gorsich et al. 2006). The enhanced expression of *ZWF1* at an early step is key to shifting the glucose metabolism in favor of pentose phosphate pathway over glycolysis (Liu et al. 2009) (Fig. 4). Consequently, all other cofactor NAD(P)H-regenerating steps involving *ZWF1, GND1, GND2,* and *TDH1* were up-regulated in the tolerant yeast. Aldehyde reduction enzyme encoding genes *ALD4, ALD6, ADH6, ADH7*, and *SFA1* displayed significantly increased transcription at the early time points in the presence of furfural–HMF complex. These accelerated NAD(P)H-dependent reductions of acetaldehyde, furfural, and HMF would generate sufficient NAD^+ and $NADP^+$, in return, to provide necessary cofactors needed for oxidative reactions or NAD(P)H regenerations by Zwf1p, Gnd1p, Gnd2p, Tdh1p, and Ald4p. Redox metabolism, in the form of interconversion of the pyridine-nucleotide cofactors NADH/NAD^+ and NADPH/$NADP^+$, plays a key role in the yeast metabolism. NADH is required in respiration and fermentative pathway in

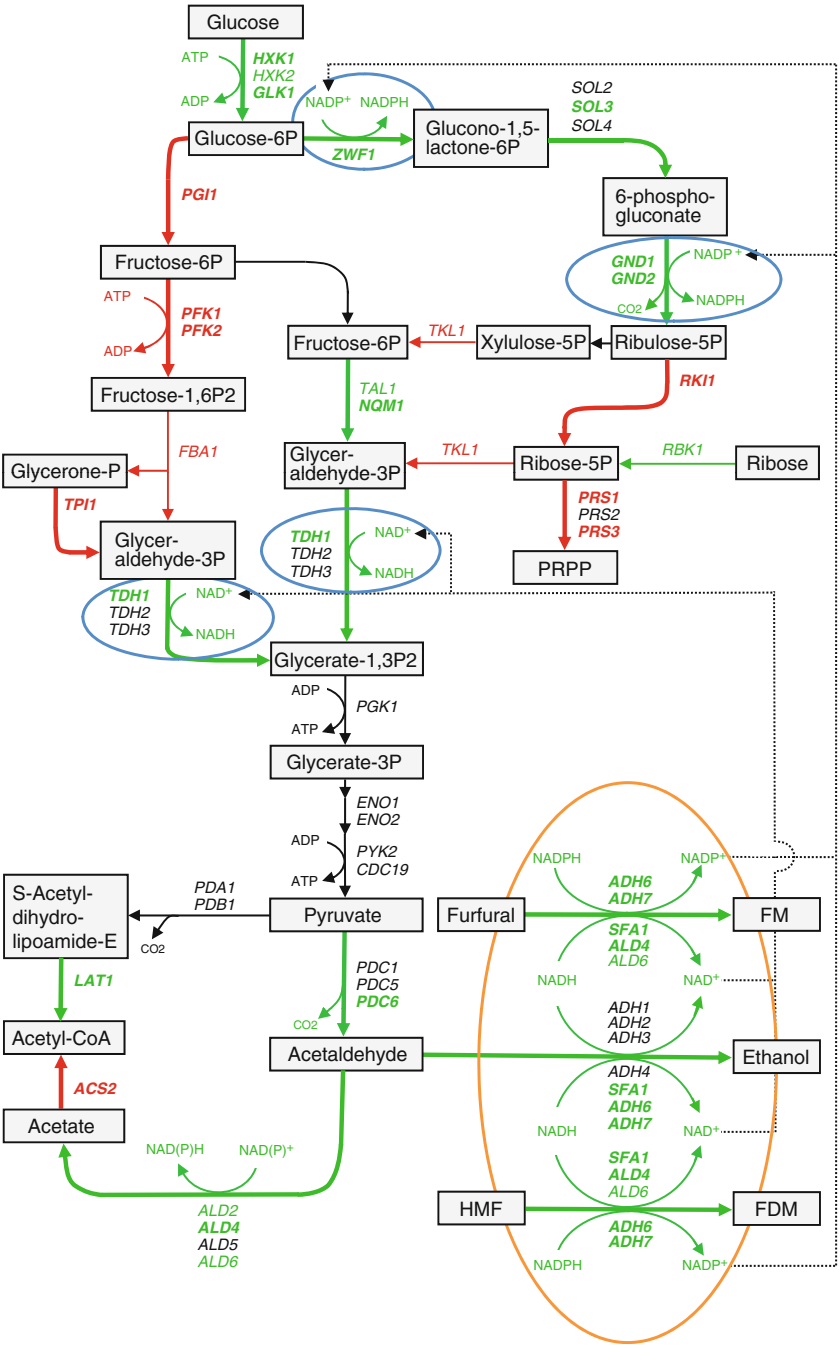

Fig. 4 A schematic illustration of glucose metabolic pathways and conversion of furfural and HMF by tolerant *Saccharomyces cerevisiae* NRRL Y-50049 inferred by metabolic profiling

conversion of pyruvate to CO_2 and ethanol (Modig et al. 2002). NADPH is mainly required for the biosynthesis of amino acids and nucleotides, and a major source of NADPH production in yeast is through the oxidative phase of pentose phosphate pathway. The up-regulated *ZWF1, SOL3, GND1*, and *GND2*, along with enhanced expressed *TDH1*, are important for NAD(P)H regenerations to supply necessary cofactors needed for acetaldehyde conversion and reduction of furfural and HMF. Thus, a $NAD(P)^+$/NAD(P)H-dependent redox balance is well maintained in the altered pathways for the in situ detoxification of furfural and HMF by the tolerant yeast.

Under the inhibitor challenge, tolerant yeast also appeared to be able to achieve NAD(P)H regeneration through a shortcut to the TCA cycle. This process involves many genes in amino acid metabolism pathways closely related to the TCA cycle, including both induced genes such as *CHA1*, *ALT1*, *PUT1*, *PUT2*, and *CAR1*, and repressed genes such as *ARG1*, *ARG3*, *ARG4*, *ARG5, ARG6*, *ARG7*, *ARG8*, *LYS4*, *LYS14*, and *LYS20* (Ma and Liu 2010). The accelerated catabolism of proline, serine, and alanine, together with the reduced biosynthesis of arginine, provides a shortcut for ATP regeneration via the TCA cycle. Thus, efficient energy metabolism can be maintained under the inhibitor stress. Apparently, enriched genetic background by aforementioned genes and a well-maintained redox balance through the reprogrammed expression responses involved in numerous pathways of the tolerant yeast strain are accountable for the acquired yeast tolerance and the detoxification of the inhibitors.

4.3 Integrated Multiple Gene Interactions

Yeasts exhibit an accelerated glucose conversion rate once they are recovered from the furfural and/or HMF challenges, compared to what would normally occur without the inhibitors (Taherzadeh et al. 2000; Liu et al. 2004, 2005). The inhibition of glucose phosphorylation, together with repression of *PFK1*, *PFK2*, *PYK2,* and *CDC19*, seemed responsible for the delayed glycolysis inhibited by furfural and HMF treatment (Liu et al. 2009). Such a delayed biological process in yeast can also be attributed to a lack of ATP, NAD(P)H, and intermediate metabolites necessary to support cell growth and reproduction (Wahlbom and Hahn-Hägerdal 2002; Fisk et al. 2006; Liu 2006). For the tolerant yeast, in addition to numerous induced expressions, gene transcription levels of *PGK1*, *ENO1*, *ENO2*, *PYK2*, *CDC19,*

Fig. 4 (continued) analysis and quantitative mRNA expression analysis compared with its wild-type strain NRRL Y-12632. *Black arrowed lines and letters* indicate normal or near-normal levels of reactions, expressions, or pathways; *green* indicates enhanced; and *red* for repressed expressions, reactions, or pathways. *Bolded lines and letters* indicate that the levels of expression and pathways are statistically significant. Key steps of enhanced NAD(P)H regenerations are *circled in blue*, and significant aldehyde reductions, *circled in orange*. Interactions of cofactor regeneration and balanced utilization pathways are linked by *dotted lines* (Liu et al. 2009)

PDA1, and *PDB1* encoding varied enzymes for pyruvate metabolisms did not exhibit repressed effect in response to the inhibitor challenge at the early stage. This allowed a smooth flow of the central metabolic pathways. Since the tolerant yeast is able to in situ detoxify the aldehyde inhibitors, with the significant reduction in the concentration of the inhibitory aldehydes, more NADPH thus generated could shift from detoxification to accelerate biosynthetic processes and cell growth. In the meantime, alcohol dehydrogenase is favored for the conversion of acetaldehyde to ethanol with sufficient NADH supply, which contributes to the accelerated glucose consumption.

It should be pointed out that many genes that initially were repressed but were able to recover to their normal functional levels after the inhibitor challenges are necessary components in these globally integrated interactions under the stress. The functions of these genes allowed the tolerant yeast to maintain balanced biological processes to complete ethanol fermentation. In the absence of such reprogrammed transcription dynamics at the genome level, continued inhibition and repression by furfural and HMF, as demonstrated by a wild-type strain, led to loss of cell function and eventual death.

5 Genomic Adaptation

The lag phase for cell growth in response to inhibitor challenges has been used as a measure of strain tolerance and to study the mechanisms of genomic adaptation (Liu et al. 2004, 2005; Liu 2006). Recently, 365 candidate genes were identified as involved in yeast adaptation and tolerance to HMF (Ma and Liu 2010). The interventional networks and interplays, as detected by gene expression regulatory networks, are complex and comprehensive. However, at least three important functional components are recognized to be mediated by several key regulators, including oxidoreductase activities, cellular transporter interactions, and protein modifications.

5.1 YAP Family and YAP1-Regulated Networks

Numerous functional encoding genes such as *ARI1, ADH6, ADH7,* and *OYE3*, as well as gene interactions involved in the biotransformation and inhibitor detoxification, are the direct driving force to reduce the HMF damage in cells for the tolerant yeast. The yeast activator protein (YAP) family contains eight transcription factors with a b-ZIP protein at the DNA-binding domain (Rodrigues-Pousada et al. 2010). Transcription factor Yap1p, the major oxidative stress regulator, acts as a sensor for oxidative molecules and activates the transcription response of antioxidant genes by recognizing Yap1p response elements (YRE), 5′-TKACTMA-3′, in the promoter region (Harbison et al. 2004; Fernandes et al. 1997; Dubacq et al. 2006). Under HMF challenged conditions, *YAP1* displayed consistently higher

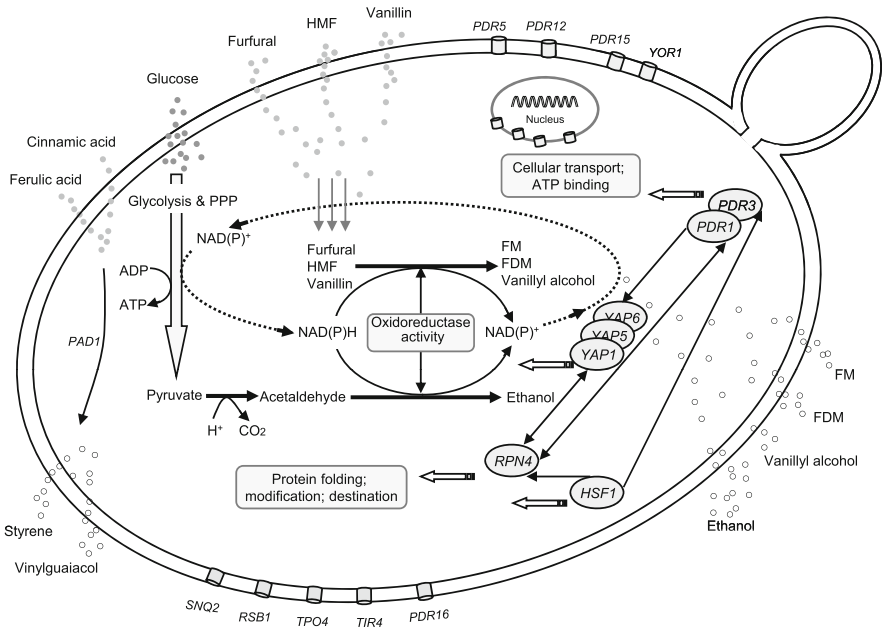

Fig. 5 A schematic diagram showing key gene regulatory elements involved in tolerance and in situ detoxification of lignocellulose hydrolysate inhibitors for *Saccharomyces cerevisiae*. Important transcription factor genes and major functional gene categories are highlighted. See text for detailed descriptions (Liu 2011)

induced abundance of at least two- to three-fold increase during the lag phase (Ma and Liu 2010). There are at least 41 HMF-induced genes possessing the YRE sequence in their promoter region. Many genes were confirmed to be regulated directly by *YAP1* or indirectly through *YAP5* and *YAP6* (Fig. 5). Most *YAP1*-regulated genes were classified in the functional categories of redox metabolism, amino acid metabolism, stress response, DNA repair, and others (Table 1). For example, the highly induced oxidoreductase genes *ADH7*, *GRE2,* and *OYE3* were found as regulons of *YAP1* (Lee et al. 2002; Haugen et al. 2004; Dubacq et al. 2006; Ma and Liu 2010). A recently characterized new aldehyde reductase gene, *ARI1,* was found to be regulated by Yap6p, which is a regulon of *YAP1* (Harbison et al. 2004; Liu and Moon 2009; Ma and Liu 2010). *ADH7* and *GRE2*, two confirmed HMF-detoxification genes encoding reductase activities, were co-regulated by Yap5p and Yap6p (Harbison et al. 2004; Workman et al. 2006; Ma and Liu 2010). A few enzyme encoding genes, for example, *ALD4* and *GRE2*, were also co-regulated by Pdr1p. In addition, *YAP1* and other YAP gene family members were shown to co-regulate numerous genes in a wide range of functional categories, such as PDR, heat shock protein, chaperones, and amino acid metabolism (Fig. 5, Table 1). In addition, multiple functions of a gene are commonly observed in tolerant yeast, and co-regulation of numerous genes can be a reflection of the multifunctions of such genes.

Single YAP gene deletion mutations are able to grow normally without HMF treatment. However, in the presence of 15 mM HMF, mutations *Δyap1, Δyap4, Δyap5,* and *Δyap6* showed delayed growth compared with their parental strain (Ma and Liu 2010). Mutant *Δyap1* displayed a 4-day-long lag phase and high sensitivity, indicating a profound defect in function affected by the *YAP1* gene. The deletion mutation of *YAP1* also showed increased sensitivity and decreased reduction activity toward coniferyl aldehyde (Sundstrom et al. 2010). This evidence supports the significant role of the YAP gene family in adaptation and tolerance to HMF. Thus, *YAP1*-regulated networks involving the functional reductase enzymes as described in a previous section are an important component for yeast tolerance and in situ detoxification of aldehyde inhibitors such as furfural, HMF, and coniferyl aldehyde. Excellent comprehensive reviews on Yap1p regulations involved in yeast stress response are available elsewhere (Herrero et al. 2008; Rodrigues-Pousada et al. 2010).

5.2 PDR Family and PDR1/3-Involved Cellular Transport Interactions

Another significant element for yeast tolerance and in situ detoxification is the PDR gene family-centered functions that are regulated by Pdr1/3p as well as other co-regulator genes such as *YAP1* and *HSF1*(Fig. 5). The PDR genes encode plasma membrane proteins and function as transporters of ATP-binding cassette (ABC) proteins. These genes mediate membrane translocation of ions and a wide range of substrates and often exhibit multiple functions in response to a large variety of unrelated chemical stresses (Mamnun et al. 2002; Moye-Rowley 2003; Jungwirth and Kuchler 2006; MacPherson et al. 2006). Many genes of the PDR family displayed consistent expressions of 3- to 30-fold increases induced by furfural and HMF treatment (Liu and sinha 2006; Song et al. 2009; Alriksson et al. 2010; Ma and Liu 2010). Gene products of these increased transcripts are characterized in a broad range of protein categories, such as drug/toxin transport for *TPO1* and *TPO4*, transport ATPase for *RSB1*, and ABC transporters for *PDR15* (Tomitori et al. 2001; Teixeira and Sá-Correia 2002; Ma and Liu 2010) (Table 2). *SNQ2*, *YOR1*, *PDR5,* and *PDR12* encoding proteins shared functions of all these three categories. These genes consist of a core set of candidate genes promoting cellular survival and adaptation to the inhibitor stress. In addition, many PDR proteins have functions as ATP-binding and chemical resistance agent.

Most of these genes have the pleiotropic drug response element (PDRE) in their promoter regions. HMF-induced transcription factor genes *PDR1* and *PDR3* regulate gene expression under a large variety of unrelated chemical stress conditions by binding to the PDRE of target genes (Mamnun et al. 2002; Moye-Rowley 2003; Jungwirth and Kuchler 2006; MacPherson et al. 2006). Both Pdr1p and Pdr3p recognize CGG triplets oriented in opposite directions to form an inverted repeat and are able to form homodimers or heterodimers to activate target gene expression

(Mamnun et al. 2002; Hellauer et al. 1996). Many induced genes regulated by Pdr1p and/or Pdr3p in this group are involved in export of both xenobiotic compounds and endogenous toxic metabolites using ABC transporters (Pdr5p, Pdr15p, Snq2p, and Yor1p), lipid composition of the plasma membrane (Rsb1p and Ict1p), export of polyamines by polyamine transporters (Tpo1p and Tpo4p), DNA repairing (Mag1p and Ddi1p), and other functions (Katzmann et al. 1995; Mahé et al. 1996; Wolfger et al. 1997; DeRisi et al. 2000; Onda et al.. 2004; Alenquer et al. 2006; Salin et al. 2008; Ma and Liu 2010). At least eight genes induced by HMF were regulated by both Pdr1p and Pdr3p. These two regulators also recognize and activate other subsets of genes. For example, Pdr3p participates in certain processes that do not involve Pdr1p, such as regulating DNA damage-inducible genes *MAG1* and *DDI1* (Zhu and Xiao 2004). Similarly, certain genes are only regulated by Pdr1p, such as *RSB1*, *ADH7,* and *PRE3* (Lee et al. 2002; Harbison et al. 2004; Kihara and Igarashi 2004). The *PDR3* promoter contains two PDREs that can be autoregulated by itself in addition to being a regulon of Pdr1p (Delahodde et al. 1995; DeRisi et al. 2000). *PDR1* and *PDR3* also demonstrated regulatory connections with a broad range of functional category genes as well as most active regulatory genes.

Gene deletion of *Δpdr1* affected reduced transcriptional abundance for many genes, including *PDR5, PDR10, PDR15, YOR1, SNQ2, ICT1, GRE2, TPO1, YMR102C,* and *YGR035C*, compared with its parental strain (Ma and Liu 2010). The mutation *Δpdr3* appeared to have a similar regulatory effect but to a lesser degree except for a clear positive impact on *PGA3*. These results confirmed the influence of *PDR1* and *PDR3* on the expression of their potential regulons. It is likely that ABC transporters play a key role to export toxic compounds such as furfural and HMF, and endogenous toxic metabolites from intracellular environment brought about by the inhibitor damage. As mentioned above, the shortcut through the TCA cycle could provide efficient energy for pumping HMF and toxic metabolites by ABC transporters under the stress.

RSB1 and *ICT1* are involved in phospholipid synthesis and transportation for membrane structure and functions that are responsible for yeast tolerance to organic solvents (Miura et al. 2000; Ghosh et al. 2008). It is possible that the induction of these PDR genes prevents the fast influx of HMF into cytoplasm and important organelles by membrane remodeling, thus, increasing the cell tolerance to HMF. *MAG1* encodes a 3-methyladenine (3MeA) DNA glycosylase (Chen et al. 1990), which acts in the first step of a multistage base excision repair pathway for the removal of lethal lesions such as 3MeA and protects yeast cells from killing by DNA-alkylating agents (Fu et al. 2008). *DDI1*, located immediately upstream of *MAG1* and transcribed in an opposite direction, encodes an ubiquitin-related protein and is involved in a DNA-damage cell-cycle checkpoint (Clarke et al. 2001). Regulatory interactions of the PDR gene family are complex, and many genes appeared to be regulated by multiple transcription factor genes involving *PDR1, PDR3, YAP1,* and *HSF1*. Regulatory roles of *PDR1* and *PDR3* to HMF challenge were suggested by computational modeling (Song and Liu 2007; Song et al. 2009).

5.3 Protein Modification Interplay Mediated by RPN4, HSF1, and Co-regulators

Degradation of damaged proteins and protein modifications consist of the third important component of the yeast tolerance and its capability for in situ detoxification. These are functioned by numerous genes such as *SHP1* and *SSA4* and regulated by transcription factor genes *RPN4* and *HSF1* as well as interplay with other closely related regulator genes such as *YAP1* and *PDR1* (Fig. 5). Chemical stress causes damage to protein conformation, leading to protein unfolding and aggregation (Goldberg 2003). Small heat shock proteins, acting as chaperones, assist in folding or refolding nascent proteins and enzymes to maintain a functional conformation (Burnie et al. 2006). For example, *HSP26* and *SSA4* encoding chaperones were significantly induced to counteract the furfural–HMF-complex damage to proteins. A deletion mutation of *SSA4* displayed a significant longer lag phase under the HMF challenge, indicating its important role in adaptation and tolerance to HMF (Ma and Liu 2010). While the presence of chaperones contributes protein protection, prolonged inhibitor stress may result in irreversible protein damages. Misfolded or damaged proteins, especially aggregated proteins, are highly toxic to cells (Goldberg 2003). Degradation of misfolded and damaged proteins by the ubiquitin-mediated proteasome pathway plays an important role in maintaining normal cell function and viability (Goldberg 2003; Wang et al. 2008, 2010). Denatured proteins are targeted via the covalent attachment of ubiquitin to a lysine side chain, and polyubiquitinated proteins are finally delivered to proteasome to be degraded. Strains with deletion mutations of these genes are sensitive to HMF, such as *OTU1* and *SHP1*. It was suggested that the degradation of proteins by the ubiquitin-mediated proteasome pathway has regulatory roles on cell cycle, metabolic adaptations, gene regulation, development, and differentiation (Glickman and Ciechanover 2002).

At least 14 ubiquitin-related and proteasome genes (*PRE1, PRE3, PRE6, PRE7, PRE10, PUP3, RPN9, RPN12, ECM29, RPT2, RPT3, RPT4, SHP1,* and *OTU1*) for protein degradation were identified in relationship to HMF adaptation (Ma and Liu 2010). These genes encoding enzymes for degradation of damaged proteins maintain cell viability and functions under the inhibitor stress. The induction of these genes was predicted to be under the control of the transcription factor Rpn4p by binding to the proteasome-associated control element (PACE, 5′- GGTGGCAAA-3′), and the PACE was found in the promoter region of most ubiquitin-related and proteasome genes induced by HMF (Mannhaupt et al. 1999; Ma and Liu 2010). The expression of *RPN4* was persistently enhanced over time during the lag phase. Rpn4p levels are regulated by 26S proteasome via a negative feedback control mechanism (Xie and Varshavsky 2001). Regulation of genes involved in DNA repair and other cellular processes is also required, such as DNA damage-inducible genes *MAG1* and *DDI1* (Harbison et al. 2004; Zhu and Xiao 2004). Interestingly, Rpn4p is a feedback regulator of *YAP1* and *PDR1* (Salin et al. 2008). This was further demonstrated by the comparative performance of the deletion mutant response to HMF. While it was able to grow and establish a culture normally

without HMF challenge, the strain harboring *Δrpn4* failed to recover in the presence of HMF (Ma and Liu 2010). These results confirmed the vital role of *RPN4* involvement in yeast tolerance. The enhanced expression of *HSF1* by HMF was consistent and statistically significantly greater. The up-regulated *HSP26* and *SSA4* for protein folding and refolding have been reported to be regulated by Hsf1p (Harbison et al. 2004; Ferguson et al. 2005; Ma and Liu 2010). Regulator gene *HSF1* is an essential gene and a positive regulator of other transcription factor genes *RPN4*, *PDR3*, *YAP5,* and *YAP6* (Lee et al. 2002; Harbison et al. 2004; Hahn et al. 2006; Workman et al. 2006). Therefore, the significant roles of *HSF1* involved in the complex co-regulation networks for the yeast tolerance cannot be underestimated.

6 Conclusion and Perspectives

It is clear that yeast tolerance and in situ detoxification of lignocellulosic hydrolysate inhibitors such as aldehydes involve complex interplays of many genes in multiple pathways at the genome scale. Functional reduction enzymes, largely involved in oxidoreductase activities, are the direct driving force in biotransformation of aldehyde inhibitors, reducing the inhibitory damages. This group of genes and their interactions are regulated by members of the yeast activator protein gene family that is led by *YAP1*. These activities are closely related to the center metabolic pathways and ethanol fermentation. Tolerant yeast can be obtained with enhanced genetic background and reprogrammed pathways to overcome furfural–HMF stress. Identification of the inhibitor functional group and the use of a structure–function strategy led to a better understanding of yeast tolerance and detoxification. Numerous members of the PDR gene family, showing consistently high levels of transcription under the inhibitor stress, are considered as tolerance candidate genes. They are actively involved in exporting xenobiotic materials and endogenous toxic metabolites and regulated mainly by *PDR1* and *PDR3*. These function-specific and multifunctional cellular transporters and ATP binding agents located at cell wall and nuclear membranes are critical for cell survival and adaptation in the presence of the inhibitors. Another necessary component of the yeast tolerance involves genes functioning in protein folding, modification, and destination that are essential to reduce degraded protein toxicity and restore protein functions. Such genes are regulated by *RPN4, HSF1,* and other co-regulators. Furthermore, all regulators rolling these three basic components are co-regulatory and interactive. However, important elements of yeast tolerance are not limited to those outlined above. As indicated by recent transcriptome and proteomic studies, general stress response and several additional significant functional categories are recognized, such as DNA repairing, oxidative stress, osmotic, and salt stress (Lin et al. 2009a, b; Ma and Liu 2010). While characterization and annotation of individual gene functions are necessary, identification of responsible functional categories and their interplays is of more importance from a global point of view.

Temporal dynamic approaches or time-course studies reveal relevant and informative insight into a life-event response and should be used more widely for yeast tolerance mechanism studies. The snap-shot kind of method needs to be limited and avoided. As demonstrated by transcription factor gene-linked regulatory interactions using systems biology approaches (Ma and Liu 2010), identification of major regulatory networks backboned with key regulators will further our understanding of the tolerance mechanisms. Fortunately, applying the advanced tools available in genomics, proteomics, metabolomics, biological engineering, and chemical engineering, a more complete understanding of molecular mechanisms and interplay for yeast tolerance at genome level may soon be reached in the near future.

Acknowledgements The author is grateful to Michael A. Cotta and Marsha Ebener for proofreading of the manuscript. This work was supported in part by NIFA National Research Initiative Award 2006-35504-17359. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Alenquer M, Tenreiro S, Sá-Correia I (2006) Adaptive response to the antimalarial drug artesunate in yeast involves Pdr1p/Pdr3p-mediated transcriptional activation of the resistance determinants TPO1and PDR5. FEMS Yeast Res 6:1130–1139

Almeida JRM, Roder A, Modig T, Laadan B, Liden G, Gorwa-Grauslund M (2008) NADH - vs NADPH-coupled reduction of 5-hydroxymethylfurfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 78:939–945

Almeida JR, Bertilsson M, Hahn-Hägerdal B, Lidén G; Gorwa-Grauslund MF (2009) Carbon fluxes of xylose-consuming Saccharomyces cerevisiae strains are affected differently by NADH and NADPH usage in HMF reduction. Appl Microbiol Biotechnol84:751–761

Alriksson B, Horváth IS, Jönsson LJ (2010) Overexpression of *Saccharomyces cerevisiae* transcription factor and multidrug resistance genes conveys enhanced resistance to lignocellulose-derived fermentation inhibitors. Process Biochem 45:264–271

Bowman MJ, Jordan DB, Vermillion KE, Braker JD, Moon J, Liu ZL (2010) Stereochemistry of furfural reduction by an aldehyde reductase from *Saccharomyces cerevisiae* that contributes to *in situ* furfural detoxification. Appl Environ Microbiol 76:4926–4932

Burnie JP, Carter TL, Hodgetts SJ, Matthews RC (2006) Fungal heat-shock proteins in human disease. FEMS Microbiol Rev 30:53–88

Chen J, Derfler B, Samson L (1990) *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of *E. coli* and is induced in response to DNA alkylation damage. EMBO J 9:4569–4575

Clarke DJ, Mondesert G, Segal M, Bertolaet BL, Jensen S, Wolff M, Henze M, Reed SI (2001) Dosage suppressors of pds1 implicate ubiquitin-associated domains in checkpoint control. Mol Cell Biol 21:1997–2007

De Rijcke M, Seneca S, Punyammalee B, Glansdorff N, Crabeel M (1992) Characterization of the DNA target site for the yeast ARGR regulatory complex, a sequence able to mediate repression or induction by arginine. Mol Cell Biol 12:68–81

Delahodde A, Delaveau T, Jacq C (1995) Positive autoregulation of the yeast transcription factor Pdr3p, which is involved in control of drug resistance. Mol Cell Biol 15:4043–4051

DeRisi J, van den Hazel B, Marc P, Balzi E, Brown P, Jacq C, Goffeau A (2000) Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Lett 470:156–160

Dubacq C, Chevalier A, Courbeyrette R, Petat C, Gidrol X, Mann C (2006) Role of the iron mobilization and oxidative stress regulons in the genomic response of yeast to hydroxyurea. Mol Genet Genomics 275:114–124

Ferguson SB, Anderson ES, Harshaw RB, Thate T, Craig NL, Nelson HC (2005) Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*. Genetics 169:1203–1214

Fernandes L, Rodrigues-Pousada C, Struhl K (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. Mol Cell Biol 17:6982–6993

Fisk DG, Ball CA, Dolinski K, Engel SR, Hong EL, Issel-Tarver L, Schwartz K, Sethuraman A, Botstein D, Cherry JM (2006) *Saccharomyces cerevisiae* S288C genome annotation: a working hypothesis. Yeast 23:857–865

Fu Y, Pastushok L, Xiao W (2008) DNA damage-induced gene expression in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 32:908–926

Gasch AP, Werner-Washburne M (2002) The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics 2:181–192

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression program in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257

Ghosh AK, Ramakrishnan G, Rajasekharan R (2008) YLR099C (ICT1) encodes a soluble Acyl-CoA-dependent lysophosphatidic acid acyltransferase responsible for enhanced phospholipid synthesis on organic solvent stress in *Saccharomyces cerevisiae*. J Biol Chem 283:9768–9775

Glickman MH, Ciechanover A (2002) The ubiquitin proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895–899

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 71:339–349

Hahn JS, Neef DW, Thiele DJ (2006) A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. Mol Microbiol 60:240–251

Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, Macisaac KD, Danford TW, Hannett NM, Tagne JB, Reynolds DB, Yoo J, Jennings EG, Zeitlinger J, Pokholok DK, Kellis M, Rolfe PA, Takusagawa KT, Lander ES, Gifford DK, Fraenkel E, Young RA (2004) Transcriptional regulatory code of a eukaryotic genome. Nature 31:99–104

Haugen AC, Kelley R, Collins JB, Tucker CJ, Deng C, Afshari CA, Brown JM, Ideker T, Van Houten B (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. Genome Biol 5:R95

Heer D, Heine D, Sauer U (2009) Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxireductases. Appl Environ Microbiol 75:7631–7638

Hellauer K, Rochon MH, Turcotte B (1996) A novel DNA binding motif for yeast zinc cluster proteins: the Leu3p and Pdr3p transcriptional activators recognize everted repeats. Mol Cell Biol 16:6096–6102

Herrero E, Ros J, Belli G, Cabiscol E (2008) Redox control and oxidative stress in yeast cells. Biochim Biophys Acta 1780:1217–1235

Jungwirth H, Kuchler K (2006) Yeast ABC transporters – a tale of sex, stress, drugs, and aging. FEBS Lett 580:1131–1138

Katzmann DJ, Hallstrom TC, Voet M, Wysock W, Golin J, Volckaert G, Moyle-Rowley WS (1995) Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae*. Mol Cell Biol 15:6875–6883

Kihara A, Igarashi Y (2004) Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry. Mol Biol Cell 15:4949–4959

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5:925–934

Laadan B, Almeida JRM, Radstrom P, Hahn-Hagerdal B, Gorwa-Grauslund M (2008) Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in *Saccharomyces cerevisiae*. Yeast 25:191–198

Larochelle M, Drouin S, Robert F, Turcotte B (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. Mol Cell Biol 26:6690–6701

Larroy C, Fernadez MR, Gonzalez E, Pares X, Biosca JA (2002a) Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. Biochem J 361:163–172

Larroy C, Pares X, Biosca JA (2002b) Characterization of a *Saccharomyces cerevisiae* NADP(H)-dependent alcohol dehydrogenase (ADHVII), a member of the cinnamyl alcohol dehydrogenase family. Eur J Biochem 269:5738–5745

Larroy C, Rosario FM, Gonzalez E, Pares X, Biosca JA (2003) Properties and functional significance of *Saccharomyces cerevisiae* ADHVI. Chem Biol Interact 143–144:229–238

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 24:151–159

Lee TI, Rinaldi NJ, Robert F, Odom DT, Bar-Joseph Z, Gerber GK, Hannett NM, Harbison CT, Thompson CM, Simon I, Zeitlinger J, Jennings EG, Murray HL, Gordon DB, Ren B, Wyrick JJ, Tagne JB, Volkert TL, Fraenkel E, Gifford DK, Young RA (2002) Transcriptional regulatory networks in *Saccharomyces cerevisiae*. Science 298:799–804

Li Bz, Yuan Yj (2010) Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 86:1915–1924

Lin FM, Qiao B, Yuan YJ (2009a) Comparative proteomic analysis for tolerance and adaptation of ethanologenic *Saccharomyces cerevisiae* to furfural, a lignocellulosic inhibitory compound. Appl Environ Microbiol 75:3765–3776

Lin FM, Tan Y, Yuan YJ (2009b) Temporal quantitative proteomics of *Saccharomyces cerevisiae* in response to a nonlethal concentration of furfural. Proteomics 9:5471–5483

Liu ZL (2011) Molecular Mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90:809–825

Liu ZL (2006) Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. Appl Microbiol Biotechnol 73:27–36

Liu ZL, Blaschek HP (2010) Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Vertes A, Qureshi N, Yukawa H, Blaschek H (eds) Biomass to biofuels: strategies for global industries. John Wiley & Sons, Ltd, West Sussex, UK, pp 17–36

Liu ZL, Moon J (2009) A novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Liu ZL, Moon J (2010) New aldehyde reductase genes of *Saccharomyces cerevisiae* Contribute in situ dexification of lignocellulose-to-ethanol conversion inhibitors.Abstract, the 14th International Biotechnology symposium and Exibition. Rimini, Italy

Liu ZL, Sinha S (2006) Transcriptional regulatory analysis reveals PDR3 and GCR1 as regulators of significantly induced genes by 5-hydroxymethy lfurfrual stress involved in bioethanol conversion for ethanologenic yeast *Saccharomyces cerevisiae*. 9th International Meeting of Microarray Gene Expression. 9:119

Liu ZL, Slininger PJ (2005) Development of genetically engineered stress tolerant ethanologenic yeasts using integrated functional genomics for effective biomass conversion to ethanol. In: Outlaw J, Collins K, Duffield J (eds) Agriculture as a producer and consumer of energy CAB International. Wallingford, UK, pp 283–294

Liu ZL, Slininger PJ (2006) Transcriptome dynamics of ethanologenic yeast in response to 5-hydroxymethylfurfural stress related to biomass conversion to ethanol. In: Mendez-Vilas A (ed) Recent research developments in multidisciplinary applied microbiology: understanding and exploiting microbes and their interactions-biological, physical, chemical and engineering aspects. Wiley-VCH, Weinheim, Germany, pp 679–684

Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31:345–352

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and 5-hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Liu ZL, Moon J, Andersh AJ, Slininger PJ, Weber S (2008a) Multiple gene mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and HMF by ethanologenic yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:743–753

Liu ZL, Saha BC, Slininger PJ (2008b) Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Wall J, Harwood C, Demain A (eds) Bioenergy. ASM Press, Washington DC, pp 17–36

Liu ZL, Ma M, Song M (2009) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282:233–244

Ma M, Liu ZL (2010) Comparative transcriptome profiling analyses during the lag phase uncover *YAP1, PDR1, PDR3, RPN4*, and *HSF1* as key regulatory genes in genomic adaptation to lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11:660

MacPherson S, Larochelle M, Turcotte B (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70:583–604

Mahé Y, Lemoine Y, Kuchler K (1996) The ATP binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids in vivo. J Biol Chem 271:25167–25172

Mamnun YM, Pandjaitan R, Mahé Y, Delahodde A, Kuchler K (2002) The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. Mol Microbiol 46:1429–1440

Martin C, Marcelo M, Almazan O, Jonsson LJ (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767–1773

Mannhaupt G, Schnall R, Karpov V, Vetter V, Feldmann H (1999) Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26s proteasomal and other genes in yeast. FEBS Lett 450:27–34

Miura S, Zou W, Ueda M, Tanaka A (2000) Screening of genes involved in isooctane tolerance in *Saccharomyces cerevisiae* by using mRNA differential display. Appl Environ Microbiol 66:4883–4889

Modig T, Liden G, Taherzadeh M (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase. Biochem J 363:769–776

Moon J, Liu ZL (2010) Protein engineering of GRE2 from *Saccharomyces cerevisiae* for enhanced detoxification of 5-hydroxymethylfurfural. Abstract. Amartean Soc Microbiol Gen Meeting San Diego, CA

Morimoto S, Murakami M (1967) Studies on fermentation products from aldehyde by microorganisms: the fermentative production of furfural alcohol from furfural by yeasts (part I). J Ferment Technol 45:442–446

Moye-Rowley WS (2003) Transcriptional control of multidrug resistance in the yeast *Saccharomyces*. Prog Nucleic Acid Res Mol Biol 73:251–279

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21:4347–4368

Nemirovskii V, Gusarova L, Rakhmilevich Y, Sizov A, Kostenko V (1989) Pathways of furfurol and oxymethyl furfurol conversion in the process of fodder yeast cultivation. Biotekhnologiya 5:285–289

Nichols NN, Dien BS, Cotta MA (2010) Fermentation of bioenergy crops into ethanol using biological abatement for removal of inhibitors. Bioresour Technol 101(19):7545–7550

Nilsson A, Gorwa-Grauslund MF, Hahn-Hagerdal B, Liden G (2005) Cofactor dependence in furan reduction by *Saccharomyces cerevisiae* in fermentation of acid-hydrolyzed lignocellulose. Appl Environ Microbiol 71:7866–7871

Onda M, Ota K, Chiba T, Sakaki Y, Ito T (2004) Analysis of gene network regulating yeast multidrug resistance by artificial activation of transcription factors: involvement of Pdr3 in salt tolerance. Gene 332:51–59

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25–33

Petersson A, Almeida JR, Modig T, Karhumma K, Hahn-Hägerdal B, Gorwa-Grauslund MF (2006) A 5-hydroxymethylfurfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. Yeast 23:455–464

Rodrigues-Pousada C, Menezes RA, Pimentel C (2010) The Yap family and its role in stress response. Yeast 27:245–258

Salin H, Fardeau V, Piccini E, Lelandais G, Tanty V, Lemoine S, Jacq C, Devaux F (2008) Structure and properties of transcriptional networks driving selenite stress response in yeasts. BMC Genomics 9:333

Song M, Liu ZL (2007) A linear discrete dynamic system model for temporal gene interaction and regulatory network influence in response to bioethanol conversion inhibitor HMF for ethanologenic yeast. Lect Notes Bioinfomatics 4532:77–95

Song M, Ouyang Z, Liu ZL (2009) Discrete dynamic system modeling for gene regulatory networks of HMF tolerance for ethanologenic yeast. IET Syst Biol 3:203–218

Sundstrom L, Larsson S, Jonsson LJ (2010) Identification of *Saccharomyces cerevisiae* genes involved in the resistance to phenolic fermentation inhibitors. Appl Biochem Biotechnol 161:106–115

Taherzadeh MJ, Gustafsson L, Niklasson C, Liden G (2000) Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:701–708

Talebnia F, Taherzadeh MJ (2006) In situ detoxification and continuous cultivation of dilute-acid hydrolysate to ethanol by encapsulated *S. cerevisiae*. J Biotechnol 125:377–384

Teixeira MC, Sá-Correia I (2002) *Saccharomyces cerevisiae* resistance to chlorinated phenoxyacetic acid herbicides involves Pdr1p-mediated transcriptional activation of TPO1 and PDR5 genes. Biochem Biophys Res Commun 292:530–537

Tomitori H, Kashiwagi K, Asakawa T, Kakinuma Y, Michael AJ, Igarashi K (2001) Multiple polyamine transport systems on the vacuolar membrane in yeast. Biochem J 353:681–688

Träff KL, Otero Cordero RR, van Zyl WH, Hahn-Hägerdal B (2001) Deletion of the GRE3 aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expression the xylA and XKSI genes. Appl Environ Microbiol 67:5668–5674

Villa GP, Bartroli R, Lopez R, Guerra M, Enrique M, Penas M, Rodriquez E, Redondo D, Jglesias I, Diaz M (1992) Microbial transformation of furfural to furfuryl alcohol by *Saccharomyces cerevisiae*. Acta Biotechnol 12:509–512

Wahlbom CF, Hahn-Hägerdal B (2002) Furfural, 5-hydroxymethylfurfrual, and acetone act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 78:172–178

Wang X, Xu H, Ju D, Xie Y (2008) Disruption of Rpn4-induced proteasome expression in *Saccharomyces cerevisiae* reduces cell viability under stressed conditions. Genetics 180:1945–1953

Wang X, Xu H, Ha SW, Ju D, Xie Y (2010) Proteasomal degradation of Rpn4 in *Saccharomyces cerevisiae* is critical for cell viability under stressed conditions. Genetics 184:335–342

Wolfger H, Mahé Y, Parle-McDermott A, Delahodde A, Kuchler K (1997) The yeast ATP binding cassette (ABC) proteins PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Lett 418:269–274

Workman CT, Mak HC, McCuine S, Tagne JB, Agarwal M, Ozier O, Begley TJ, Samson LD, Ideker T (2006) A systems approach to mapping DNA damage response pathways. Science 312:1054–1059

Xie Y, Varshavsky A (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc Natl Acad Sci USA 98:3056–3061

Zhu Y, Xiao W (2004) Pdr3 is required for DNA damage induction of MAG1 and DDI1 via a bi-directional promoter element. Nucleic Acids Res 32:5066–5075

Genetics and Regulation of Glycogen and Trehalose Metabolism in *Saccharomyces cerevisiae*

Jean Marie François, Thomas Walther, and Jean Luc Parrou

Contents

1 Introduction 30
2 Metabolic Pathways 30
2.1 The Glycogen Metabolic Pathway 30
2.2 The Trehalose Metabolic Pathway 36
2.3 UDP-Glucose Partitioning 38
3 Nutrients, Stress, and Growth Control of Glycogen and Trehalose 39
4 Biological Function of Storage Carbohydrates in Yeast 42
4.1 Function as Energy and Carbon Stores 42
4.2 Specific Function of Trehalose as a Stress Protectant 44
5 The Role of Tps1/ Trehalose-6-Phosphate in Carbon and Energy Metabolism 45
6 Conclusion and Perspectives 47
References 48

Abstract Glycogen and trehalose are two important glucose stores of the yeast *Saccharomyces cerevisiae*, and the content of which varies strongly and rapidly in response to changing environmental conditions. Although the metabolic pathways of these two glucose stores have been studied for decades, recent biochemical and molecular studies have unraveled unexpected metabolic features, such as the ability to accumulate glycogen in the absence of glycogenin, the demonstration that acid trehalase encoded by *ATH1* is localized at the cell surface instead of the vacuole and

J.M. François (✉) • J.L. Parrou
INSA UPS INP & INRA, University of Toulouse, 135 Ave de Rangeuil, F-31077 Toulouse, France

CNRS-UMR 5504, F-31077 Toulouse, France
e-mail: fran_jm@insa-toulouse.fr

T. Walther
INSA UPS INP & INRA, University of Toulouse, 135 Ave de Rangeuil, F-31077 Toulouse, France

INRA-UMR 792 Ingénierie des Systèmes Biologiques et procédés, Toulouse, France

CNRS-UMR 5504, F-31077 Toulouse, France

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_2,

allows cells to grow on trehalose. It is also clearly demonstrated that glycogen and trehalose pathways are subject to hierarchical control dependent on major nutrient-sensing protein kinases, namely TOR, PKA, Snf1 kinase homologous to mammalian AMP-activated protein kinase (AMPK), Pho85p, and the energy sensor Pas kinase. The sophisticated control mechanisms highlight the importance of these two glucose stores in the context of growth and cell cycle of the yeast.

1 Introduction

The budding yeast *Saccharomyces cerevisiae* accumulates two types of glucose stores, glycogen and trehalose. Glycogen is a high molecular mass branched polysaccharide of linear α-(1,4)-glucosyl chains with α-(1,6)-linkages, whereas trehalose is a nonreducing disaccharide composed of two α-(1,1)-linked glucose molecules. The content of these two glucose stores varies strongly and rapidly in response to changing environmental conditions, which emphasizes their role as energy and carbon resources in yeast cells. Detailed biochemical and molecular studies over the past 10 years have shown that both glycogen and trehalose metabolic pathways are subject to hierarchical control dependent on major nutrient-sensing protein kinases, namely TOR, PKA, Snf1 kinase homologous to mammalian AMP-activated protein kinase (AMPK), Pho85p, and the energy sensor Pas kinase. This chapter provides an overview of the genetic and metabolic control of storage carbohydrate metabolism and discusses these mechanisms in the context of growth and cell cycle of the yeast *S. cerevisiae*. For enzymatic systems participating in glycogen or trehalose metabolism, readers can refer earlier reviews (Lomako et al. 2004; Gancedo and Flores 2004; Parrou et al. 2005).

2 Metabolic Pathways

This section describes pathways and regulation of glucose storage in the yeast *Saccharomyces cerevisiae*. It includes new and unexpected features underlined using combined approaches of genomics, genetics, and proteomics by researchers over the last 10 years.

2.1 The Glycogen Metabolic Pathway

Glycogen is a highly branched polysaccharide of linear α-(1, 4)-glucosyl chains with α-(1, 6)-linkages. Each linear chain has an average length of 13 glucose units and contains two branching points by means of α-(1, 6) glycosidic bonds (Fig. 1). This structural organization results in a spherical shape of the glycogen molecule

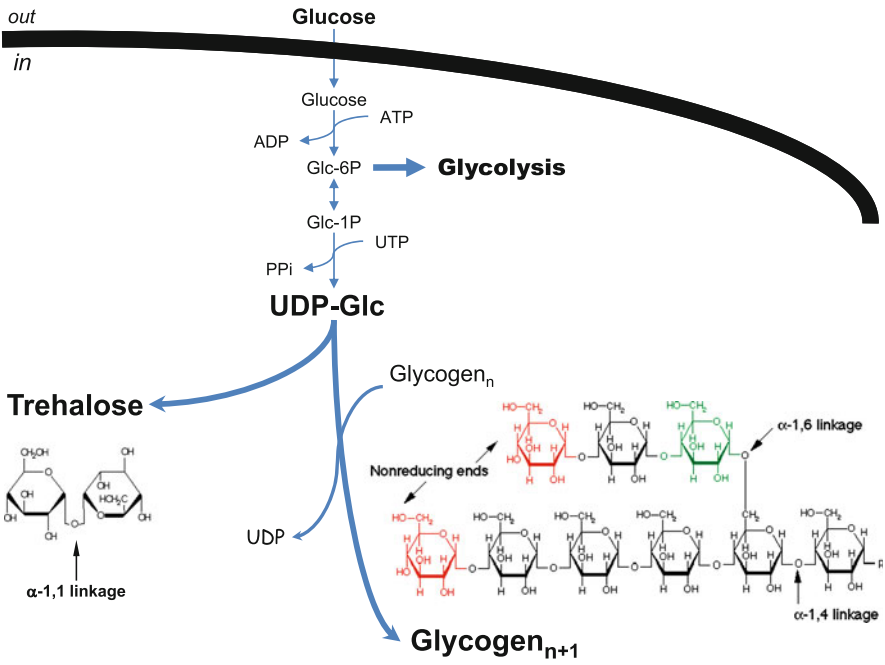

Fig. 1 Structures of glycogen and trehalose and their metabolic routes from glucose in the yeast *S. cerevisiae*

reaching a molecular mass $>10^6$ Da (Melendez et al. 1999). As it is the case for the production of any polymers, the formation of glycogen requires enzymes for initiation, elongation, and ramification. The initiation step is carried out by a protein denoted "glycogenin" which preferentially uses UDP-Glc but can accept CDP-Glc or TDP-Glc as substrates (Alonso et al. 1995a) to produce a short α-(1,4)-glucosyl chain covalently attached to a tyrosine residue by autoglucosylation activity. This initiation step is specific for all eukaryotic cells and has not been identified in bacteria in which the glycogen synthase is responsible for both initiation and elongation (Ugalde et al. 2003). In yeast, glycogenin is encoded by two genes, *GLG1* and *GLG2* (Cheng et al. 1995; Cheng et al. 1995). However, loss of function of these genes did not result in a complete glycogen deficiency but in a stochastic accumulation of glycogen particles in some individual colonies (Torija et al. 2005). In addition, the occurrence of these glycogen positive *glg1 glg2* mutant colonies is strongly enhanced by the presence of a hyperactive glycogen synthase or upon deletion of *TPS1*, encoding a subunit of the trehalose synthase complex. Altogether, these results contradict previous claims that glycogenin is essential for glycogen biogenesis in eukaryotic cells (Lomako et al. 2004) and favor the idea that the initiation step can take place using alternative primers whose synthesis and/or distribution may be controlled by epigenetic silencing (Torija et al. 2005). On the other hand, overproduction of glycogenin does not lead to hyperaccumulation of

glycogen (Cheng et al. 1995), indicating that these proteins are likely reiteratively used in the glycogen synthesis process. Details of this reiteration process remain to be demonstrated.

The elongation step is catalyzed by glycogen synthase that operates by the successive addition of α-1,4-linked glucose residues to the nonreducing end of glycogen, using UDP-Glc as the donor substrate. The yeast *S. cerevisiae* contains two genes, *GSY1* and *GSY2*, encoding two glycogen synthases (Fig. 2) that are 80% identical at the protein level and share 50% similarity with the mammalian muscle enzyme. Gsy2p was shown to be the predominant glycogen synthase as indicated by a 90% reduction in both enzyme activity and glycogen levels in a *gsy2Δ* mutant growing on glucose (Farkas et al. 1991; Farkas et al. 1990). However, under other conditions such as during growth on galactose, Gsy1p appears to be as important as Gsy2p in glycogen accumulation (JL Parrou & J François, personal communication).

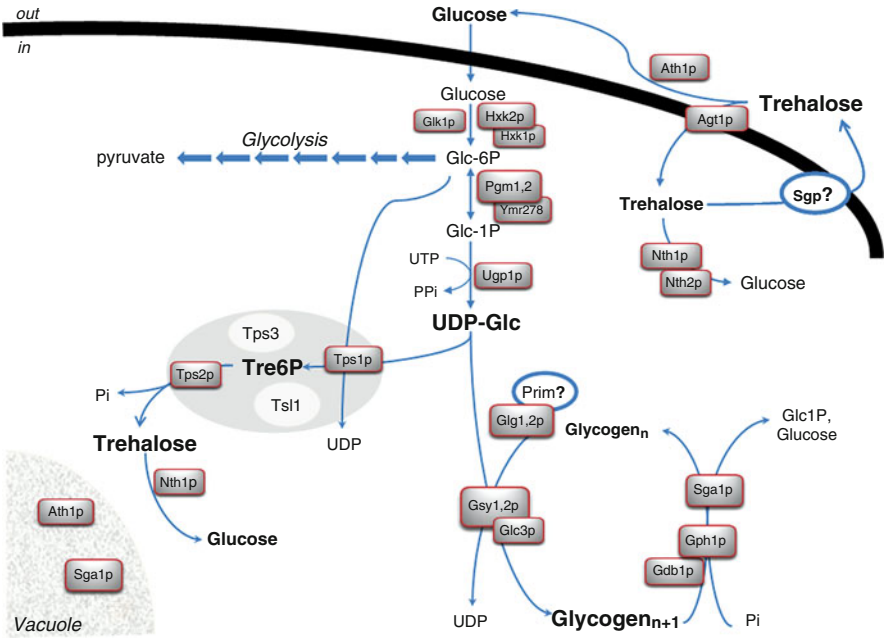

Fig. 2 A schematic illustration of the metabolic pathways for glycogen and trehalose biosynthesis and biodegradation and for trehalose assimilation in the yeast *S. cerevisiae*. Putative or yet uncharacterized proteins in the pathways are outlined. Abbreviation of the enzyme name: Hxk2p, hexokinase II; Hxk1p, hexokinase I; Glk1p, glucokinase; Pgm1,2p, phosphoglucose mutase isoform 1 and 2; Ymr278p, homologous to phosphoglucomutase; Ugp1p, uridylylglucose pyrophosphorylase; Glg1,2p, glycogenin isoform 1 & 2; Gsy1,2p, glycogen synthase isoform 1 & 2; Glc3p, glycogen branching enzyme; Gph1p, glycogen phosphorylase; Gdb1p, glycogen debranching enzyme; Sga1p, amylo (1 → 4), (1 → 6) glucosidase; Ath1p, acid trehalase or extracellular trehalase; Agt1p, α-methylglucose transporter; Nth1, 2p, neutral trehalase isoform 1 & 2; Sgp1p, putative sugar permease; Tps1p, trehalose-6-phosphate synthase; Tps2p; trehalose-6-P phosphatase; Tps3p & Tsl1p, regulatory subunit of the trehalose synthase complex

Elongation of glucosyl chains in glycogen is followed by the branching step catalyzed by an amylo α-(1,4), α-(1,6)-transglucosidase (branching enzyme) encoded by *GLC3* (Thon et al. 1992). This enzyme transfers a terminal stretch of seven glucose residues from the linear α-(1, 4) glucosyl chain to another linear chain making an α-(1, 6) bond between glucosyl units deeper in the molecule.

Like in mammals, metabolic regulation of glycogen synthesis in yeast is brought about through allosteric control of glycogen synthase by Glc6P and by reversible covalent phosphorylation. The nonphosphorylated, highly active form of glycogen synthase is insensitive to Glc6P, whereas the phosphorylated, less active form is highly dependent on the presence of the sugar phosphate. Thus, measurement of glycogen synthase activity in the absence and in the presence of Glc6P gives a direct value of the ratio between these two interconvertible forms (Francois and Hers 1988; Pederson et al. 2000). Mutagenesis studies in yeast (Pederson et al. 2004; Pederson et al. 2000) identified two conserved Arg clusters (Arg579/580/582/583 and Arg586/587/588/591) that are part of the allosteric control site for Glc6P. Refinement of the crystal structures corresponding to the basal activity state and glucose-6-phosphate activated state of yeast glycogen synthase-2 allowed showing that the enzyme is assembled into an unusual tetramer by an insertion unique to the eukaryotic enzymes, and this subunit interface is rearranged by the binding of glucose-6-phosphate, which frees the active site cleft and facilitates catalysis. Two arginine residues at positions 583 and 587 are shown to be responsible for the enzyme's response to control by Glc6P, while the other Arg residues are implicated in the phosphorylation response of Gsy2p (Baskaran et al. 2010). Glycogen synthase possesses three phosphorylation sites (Ser-650, Ser-654, and Thr-667) at its C-terminus, which is in accordance with a maximum of 3 moles phosphate/mol protein incorporated in the purified inactive glycogen synthase (Peng et al. 1990) and with the observation that the removal of the C-terminus by mild proteolysis results in a fully active, Glc6P-insensitive form of Gsy2p (Hardy and Roach 1993). The cyclin-dependent kinase Pho85 and the PAS kinase encoded by *PSK1* and *PSK2* (Rutter et al. 2002) are the two so far identified kinases that can directly phosphorylate Gsy2p *in vitro* and *in vivo* (Huang et al. 1998; Rutter et al. 2002). The cAMP-dependent protein kinase (PKA) is not effective on this enzyme (Hardy and Roach 1993) in spite of the fact that several mutants impaired in the PKA activity exhibit strong alteration of their glycogen content. The action of Pho85p on Gsy2p requires association of Pho85p with its cyclin partners Pcl8p and Pcl10p. This protein complex facilitates phosphorylation of Gsy2p at Ser-654 and Thr-667 (Wilson et al. 1999) (Fig. 2), and failure of this phosphorylation event results in a hyperactive glycogen synthase and higher glycogen content of the cells (Wang et al. 2001; Timblin et al. 1996). On the other hand, the PAS kinase only phosphorylates the Ser-654 *in vivo*. This phosphorylation is physiologically relevant since a mutant defective in this kinase has a higher Glc6P-dependent glycogen synthase activity (Rutter et al. 2002). The reversibility of a phosphorylation event is ensured by protein phosphatases, which remove the covalently bound phosphate from Ser/Thr. On Gsy2p, this task is mainly taken over by type I Ser/Thr protein phosphatase encoded by *GLC7*, which is targeted to the glycogen synthase by a specific targeting

subunit encoded by *GAC1* (Francois et al. 1992; Skroch Stuart et al. 1994). A tripartite interaction of Gac1p with Glc7p and Gsy2p has been demonstrated, and these interactions are necessary for a productive and complete dephosphorylation of glycogen synthase (Wu et al. 2001). In addition to Gac1p, two proteins encoded by *PIG1* and *PIG2* isolated by two-hybrid screen with Gsy2p as the bait (Cheng et al. 1997) may participate in the control of glycogen synthesis since a *gac1 pig1 pig2* triple mutant shows a more severe glycogen defect than a *gac1* single mutant, whereas a *pig1 pig2* double mutant does not show any glycogen defect (Cheng et al. 1997; J. Francois, unpublished data). Besides the major role of the type I Ser/Thr protein phosphatase, type 2A protein phosphatase has been shown to exert a minor effect on glycogen synthase, but this control is likely to take place at the transcriptional level (Posas et al. 1993) (see below).

In summary, the activity of glycogen synthase is controlled by the dynamic equilibrium between the active, nonphosphorylated form and the less active, phosphorylated form of the enzyme. Whether the active or less active form of glycogen synthase is more abundant in the cells depends on the relative activities of kinases and phosphatases that are acting on Gsy2p. In addition to its role as an allosteric activator of glycogen synthase, Glc6P likely orchestrates the transition between the different phosphorylation states of Gsy2p by stimulating dephosphorylation and inhibiting phosphorylation of the enzyme (Francois and Hers 1988; Pederson et al. 2004; Baskaran et al. 2010). Therefore, one can expect that any condition leading to dramatic changes in Glc6P should have a direct impact on glycogen synthesis (Fig. 3). This hypothesis is actually supported by mutants defective in phosphoglucose isomerase (*pgi1*), in mutant with a reduced activity of the glycolytic 6-phosphofructokinase (*pfk2*) as they contain both higher Glc6P and higher glycogen levels than the wild type on glucose (Corominas et al. 1992; Huang et al. 1997), as well as in *tps1* mutant defective in trehalose synthesis that also exhibit very high levels of hexose monophosphates (J François, Th Walter, and JL Parrou, unpublished results).

The biodegradation of glycogen in yeast occurs in the cytosol by the sequential actions of glycogen phosphorylase and glycogen debranching enzymes encoded by *GPH1* (Hwang et al. 1989) and *GDB1* (Teste et al. 2000), respectively, which degrade glycogen to glucose-1-P and glucose (Fig. 2). Like in mammals, the yeast glycogen phosphorylase (Gph1p) is activated by phosphorylation, and this phosphorylation occurs on a single threonine residue (Thr^{31}) of the protein (Lin et al. 1995). Since Gsy2p and Gph1p exist as interconvertible forms in the cells, the balance of the two forms depends upon the stringent of the relative activity of the kinases and phosphatases. Unlike for glycogen synthase, there is no technical means to determine the proportion of the two Gph1p forms *in vivo*, although it is feasible with the mammalian cells for which the dephosphorylated, inactive form is highly sensitive (and stimulated by) to AMP (Fletterick et al. 1986). In addition, the protein kinases and protein phosphatases implicated in the regulation of yeast Gph1p regulation have not yet been fully understood. Recent data indicated the implication of the Pho85-Pcl6p/Pcl7p complex in controlling the phosphorylation state of Gph1p. However, this implication was not direct but mediated through the

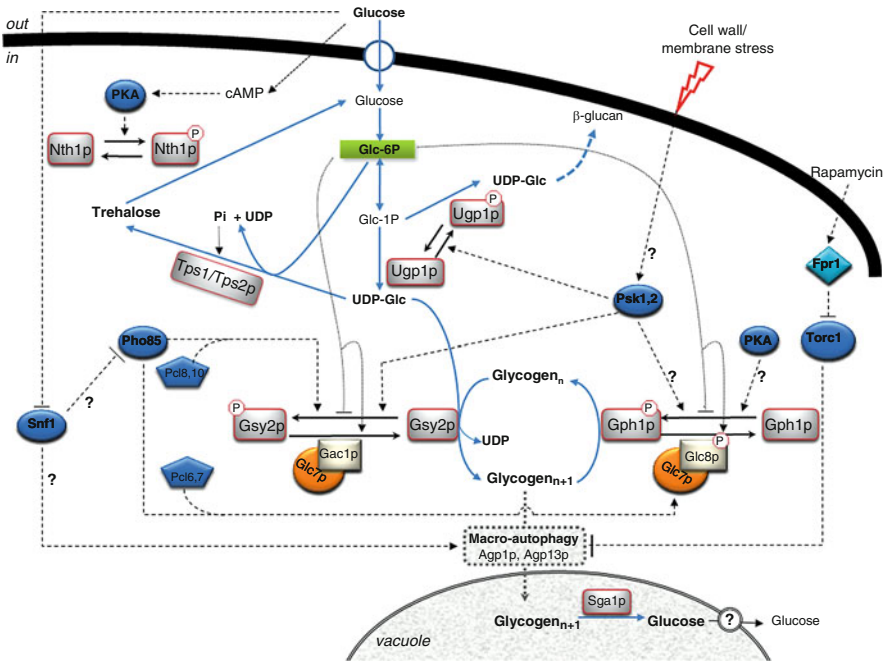

Fig. 3 Scheme of the posttranslational (metabolic) control of glycogen and trehalose in the yeast *S. cerevisiae*. *Question marks* indicate mechanisms of actions either uncharacterized or uncertain. See text for detailed explanations

phosphorylation of Glc8p. This latter protein interacts with the protein phosphatase Glc7p to form a Glc7–Glc8p complex, which in turn dephosphorylates and hence inactivates Gph1p (Wilson et al. 2005). Therefore, and contrary to expectation, the effect of Pho85p is also to inactivate Gph1p as in the same time this kinase inactivates Gsy2p by direct phosphorylation. This result merits further investigation since it contradicts the fact that two enzymes are controlled by an on/off mechanism, posing that glycogen synthase be active when glycogen phosphorylase is inactive and vice versa (Francois and Parrou 2001). The PKA has been reported to phosphorylate Gph1p *in vitro* (Lin et al. 1995) but has not been verified *in vivo*. On the other hand, the crystal structure analysis of phosphorylated and nonphosphorylated Gph1p bound to Glc6P revealed that this metabolite serves as a dephosphorylation facilitator by modifying the accessibility of the phosphorylation site to protein phosphatases (Lin et al. 1996). This finding supports the role of Glc6P as a major effector controlling glycogen phosphorylase activity *in vivo* (Fig. 3).

The other mechanism for glycogen breakdown involves an amylo (1,4), (1,6) glucosidase encoded by *SGA1* that releases glucose as the final product (Colonna and Magee 1978; Clancy et al. 1982). Initially thought to be expressed only during sporulation (Clancy et al. 1982; Chu et al. 1998), *SGA1* has now been found to be induced in late stationary phase or under starvation conditions (Teste et al. 2009).

The encoded protein is localized in vacuoles and serves to hydrolyze glycogen particles that have been imported into vacuole by the autophagy process during growth on glucose. Thus, the vacuolar glycogen pool is protected from degradation by the cytosolic glycogen phosphorylase and takes place only under extreme growth conditions (Wang et al. 2001). The observation that impairment of the vesicular trafficking or of the vacuole formation resulted in hypoaccumulation of glycogen in cells at the stationary phase or under starved conditions argues in favor of this model (Wilson et al. 2002b).

It should be pointed out that glycogen has been recognized as the first biological fractal structure at the molecular level. Fractal objects are complex structures built by an iterative process, which is the case for the glycogen molecule (Alonso et al. 1995b). To successfully produce this fractal structure, the following rules have to be obeyed: (i) the branching activity must be in excess over the synthase such that a new branch is made when it is physically possible, (ii) the growth of glycogen must be favored in the inner growing chains to avoid excessive external growth, and (iii) glycogen phosphorylase should exhibit activities even during the biosynthesis of the polymer in order to correct any mistake under the abovementioned conditions (Melendez et al. 1999). While two out of the three conditions have received experimental evidence (Wilson et al. 2004), the role of glycogen phosphorylase in the synthesis of the fractal glycogen structure remains to be proved.

2.2 The Trehalose Metabolic Pathway

Trehalose is a disaccharide made of two glucose units linked by an α-1 → 1 bond (Fig. 1). The metabolic pathways for synthesis, mobilization, and assimilation of this disaccharide are depicted in Fig. 2. At least five different biosynthetic pathways are known for trehalose synthesis (Avonce et al. 2006). The most widely distributed pathway in nature, present in fungi, consists of two consecutive enzymatic reactions employing a trehalose-6-phosphate-synthase (TPS) enzyme, producing the intermediate trehalose-6-phosphate (Tre6P), and a Tre6p-phosphatase (TPP) enzyme. In filamentous fungi and yeasts, the two activities are borne on a single protein complex, whereas in bacteria, they exist as two separated entities. A recent evolutionary study on trehalose biosynthesis genes provided evidence that the formation of bifunctional protein complexes took place already in some group of bacteria and archea, but the physiological consequence of this protein fusion is still unclear (Avonce et al. 2010). In *S. cerevisiae*, the TPS/TPP complex is encoded by *TPS1* and *TPS2*, respectively, and contains two additional subunits encoded by *TPS3* and *TSL1* that are apparently not present in other fungal TPS/TTP (Kwon et al. 2003; Avonce et al. 2006). These two subunits show high degree of similarity and may function as stabilizer of the complex as suggested by the fact that a *tps3 tsl1* double mutant has a reduced TPS activity and trehalose content (Reinders et al. 1997; Bell et al. 1998). The loss of *TPS1* not only abolishes the synthesis of trehalose but also causes several other metabolic disorders that will be detailed below. Also, the

deletion of *TPS2* results in a temperature-sensitive growth phenotype, which has been attributed to an excessive accumulation of Tre6P since a suppressor of this phenotype was found to be *PMU1* encoding a putative phosphomutase. Overexpression of *PMU1* reduced levels of Tre6P and converted it into yet uncharacterized intermediates (Elliott et al. 1996). In contrast to enzymes of the glycogen pathway, the TPS/TPP is not the subject of reversible phosphorylation. The Tps1p subunit is highly sensitive to inhibition by Pi, which acts as a noncompetitive inhibitor to both Glc6P and UDP-Glc ($K_i \sim 2$ mM) (Vandercammen et al. 1989; Londesborough and Vuorio 1993), whereas Tps2p requires the presence of Pi for full activity. On the other hand, Fru6P acts as an allosteric effector, reducing the Km for Glc6P from around 5 to 1.5 mM. Taking into account these enzymatic data, the *in vivo* rate of Tre6P synthesis is actually largely determined by the availability of Glc6P and UDP-Glc as substrates and by levels of its main allosteric effectors, Fru6P and Pi (Vandercammen et al. 1989; Londesborough and Vuorio 1993), besides the fact that the TPS complex is also subject to repression by glucose (Neves et al. 1991; Winderickx et al. 1996). This may thus explain the rapid accumulation of Tre6P that takes place upon glucose addition to respiring yeast cultures as under this condition, there is a transitory increase of Glc6P and Fru6P, accompanied by a drop of Pi triggers which leads to imbalance of Tps1p and Tps2p activity (Walther et al. 2010). However, trehalose accumulation during stationary phase is not accompanied by a noticeable increase of Tre6P (J. François, unpublished data), which indicates that both trehalose 6-P synthase and phosphatase functioned at the same rate.

In the yeast *S. cerevisiae*, two types of trehalase, distinct in their optimal pH and localization, can hydrolyze trehalose into glucose. *NTH1* encodes a cytosolic trehalase that is optimally active at neutral pH with a relatively high K_m (5–35 mM) for trehalose (Londesborough and Varimo 1984; App and Holzer 1989). A relevant regulatory property of Nth1p is to be activated by phosphorylation. To date, the PKA is the sole protein kinase that has been reported to directly phosphorylate this protein (Fig. 3). Interestingly, the Nth1p harbors eight putative PKA-dependent phosphorylation sites (Wera et al. 1999), but only Ser^{21} and Ser^{23} have been shown to be phosphorylated *in vivo* (Ficarro et al. 2002). In addition, complete activation of Nth1p requires the binding with the 14-3-3 protein encoded by *BMH1*/*BMH2* on phosphorylated Ser^{21} (Panni et al. 2008). Yeast possesses a second functional trehalase encoded by *NTH2* that is 77% identical to Nth1p (Jules et al. 2008). Little is known about the kinetic properties and regulation of the second trehalase, except that it has been implicated in trehalose mobilization in late stationary phase of growth on glucose or upon growth recovery from heat and saline stress (Nwaka et al. 1995; Jules et al. 2008; Garre and Matallana 2009).

Another hydrolase acting on trehalose is encoded by *ATH1*. Strong experimental evidence shows that this trehalase has a dual localization, both at the cell surface and in the vacuole. However, only the cell-surface localized enzyme was found to be active and able to hydrolyse extracellular trehalose (Jules et al. 2004; He et al. 2009). Hence, this localization can account for its requirement for the growth on trehalose as a sole carbon source (Nwaka et al. 1996; Jules et al. 2004) (Fig. 2).

The cell-surface localization of Ath1p is likely mediated by the classical secretion "Sec" pathway, despite the fact that the protein does not harbor any secretion signal (He et al. 2009); S.He and JL Parrou, unpublished data), whereas the delivery of Ath1p to the vacuole follows the multivesicular body pathway (MVB) (Huang et al. 2007). The function of this vacuolar-localized Ath1p is unknown. Since constraining the enzyme into vacuolar impairs the growth on trehalose, no evidence for vacuolar import of trehalose by the autophagy process can be done as it has been shown for glycogen (Jules et al. 2008) (Fig. 2). As a result, it is proposed to replace the terms of neutral and acid trehalase by "cytosolic" and "extracellular" trehalases as they are more adequate to describe the localization and the function of these two enzyme forms (Parrou et al. 2005).

As mentioned above, trehalose can be assimilated as an exogenous carbon source by several fungi, including the yeast *S. cerevisiae* (Parrou et al. 2005). In addition to the Ath1-dependent pathway (Fig. 2), a second route that couples the high-affinity trehalose H^+-symporter encoded by *AGT1* (Plourde-Owobi et al. 1999) with the neutral trehalase encoded by *NTH1* can facilitate cell growth on trehalose (Jules et al. 2004). However, this second pathway is not functional in *mal*$^-$ strain because *AGT1* expression is dependent upon the *MAL* system (Han et al. 1995) or is weakly effective even in Mal$^+$ strain since Agt1p rapidly loses activity during growth on trehalose (Jules et al. 2004). It is noteworthy that the growth on trehalose is strictly respiratory (Jules et al. 2005) and thus subject to the so-called Kluyver effect, i.e., the inability to ferment a sugar even under anaerobic conditions (Fukuhara 2003). This effect is likely due to the rate-limiting activity of Ath1p since the growth rate can be increased to a maximum of threefold by overexpression of *ATH1* (Jules et al. 2005; He et al. 2009). However, no further increase in growth rate could be obtained even after a 20-fold increase in the expression of Ath1p, suggesting that other limiting steps may exist that prevent cells to ferment trehalose.

2.3 UDP-Glucose Partitioning

UDP-glucose is a donor of glucose units at the crossroads between several pathways, including glycogen and trehalose, cell wall β-glucan, and glycosylation of proteins. The production of UDP-Glc is catalyzed by UDP-glucose pyrophosphorylase encoded by *UGP1* (Daran et al. 1995). Significant reduction of UDP-Glc levels by reducing the activity of Ugp1p was accompanied by a significant decrease in glycogen and trehalose production, whereas levels of cell-wall β-glucan were slightly altered, raising the hypothesis that UDP-glucose could be channeled toward the synthesis of β-glucan (Daran et al. 1997). A partitioning of glucose toward β-glucan and away from glycogen (Smith and Rutter 2007) demonstrated that Ugp1p is phosphorylated on Ser^{11} by the PAS kinase with the consequence that the phosphorylated enzyme is targeted to the cell periphery to favor glucan synthesis, while its catalytic activity is not affected (Fig. 3). Therefore, the inability to phosphorylate Ugp1p or the deletion of *PSK1* and *PSK2* leads to elevation of

glycogen and renders the cells hypersensitive to cell wall perturbing agents likely because of a reduction of β-glucan.

3 Nutrients, Stress, and Growth Control of Glycogen and Trehalose

It is well established that levels of glycogen and trehalose in yeast cells vary significantly according to growth, nutrients, and stress conditions (e.g., osmotic, saline, and heat shock (Francois and Parrou 2001)). These variations are accounted largely to the main nutrient-sensing pathways PKA, TOR, and SNF1 (Fig. 4). As recently illustrated by microarrays analyses of starved cells challenged with nutrient repletion (Slattery et al. 2008), the transcription response, which is strongly repressive for the glycogen and trehalose-related genes, is largely dependent on the cAMP/PKA pathway. At present, this repressive effect exerted by the PKA is explained by at least three modes of action. First, and likely the most effective mechanism, is to restrict Msn2/4p in the cytosol, which is facilitated by the PKA-dependent phosphorylation

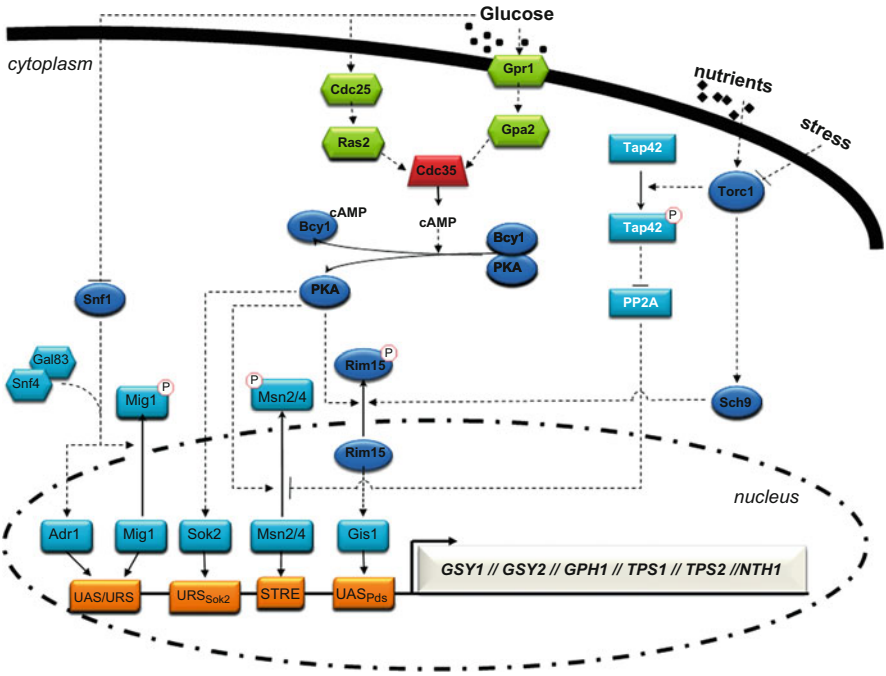

Fig. 4 A schematic illustration of the transcriptional control of glycogen and trehalose by the nutrient-sensing pathways dependent on the PKA, TORC1, and SNF1 kinases in the yeast *S. cerevisiae*. Genes given as targets in these pathways are those encoding principal enzymes in the synthesis and degradation of the two glucose stores. See text for detailed explanations

of this protein (Gorner et al. 2002). Consequently, this restriction prevents the transcription activation of glycogen and trehalose-related genes (to which can be included *PGM2* and *UGP1*, two genes required for the production of UDP-Glc) that normally takes place by DNA binding of Msn2/4p to the STRE elements (CCCCT) present in several copies in the promoter of these genes (Ni and LaPorte 1995; Parrou et al. 1999b; Winderickx et al. 1996; Parrou et al. 1999a; Sunnarborg et al. 2001; Zahringer et al. 2000). The second mechanism implicates the transcriptional repressor Sok2p (Ward et al. 1995) since all important glycogen and trehalose-related genes harbor a consensus motif for *SOK2* in their promoter region. It was reported that overexpression of *SOK2* reduced expression of *GAC1* (Ward et al. 1995); J. François, unpublished data), whereas *SOK2* deletion partially released *GSY2* repression in a mutant with a high PKA activity (Enjalbert et al. 2004). A third pathway by which the PKA exerts its repressing effect is through the blockage of the Rim15-Gis1p cascade. The latter pathway mediates its effects through an upstream activating sequence (UAS_{PDS}) that is present in most of the glycogen and trehalose-related genes. However, this cascade is only operative in stationary phase cells or when cells enter into quiescent G0 state (Pedruzzi et al. 2000; Pedruzzi et al. 2003).

Evidence has been accumulated that the TOR (target of rapamycin) pathway, through its TORC1 complex (complex made of Tor1p or Tor2p with three other partners, Kog1p, Lst8p, Tco89p; see (De Virgilio and Loewith 2006 for a review), also affects storage carbohydrates as shown by rapamycin-induced glycogen and trehalose accumulation in yeast cells growing on glucose (Barbet et al. 1996). This accumulation is accompanied by upregulation of the glycogen and trehalose genes (Zurita-Martinez and Cardenas 2005). This upregulation can be explained by the effect of rapamycin to induce nuclear localization of Msn2/4p via inhibition of TORC1 (Santhanam et al. 2004). Therefore, TORC1 may negatively control storage metabolism through a signaling pathway involving phosphorylation of Tap42p, which in turn inhibits Ser/Thr protein phosphatase 2A by direct binding. Consequently, this latter protein is no longer able to dephosphorylate Msn2p, which therefore remains sequestered in the cytoplasm (Zaman et al. 2008). This mechanism can therefore account for previous reports showing effects of this type 2 phosphatase on glycogen levels (Clotet et al. 1995). Additionally, TORC1 has been shown to negatively control Rim15-Gis1p cascade through the protein kinase Sch9 (De Virgilio and Loewith2006). Altogether, these data bring into light a converging effect of the two main nutrient-sensing pathways, PKA and TOR, on Msn2/4p to regulate expression of glycogen and trehalose genes. Hence, any changes in the balance of the activities of PP2A and PKA may directly impact the expression levels of these genes and eventually on the attendant metabolism. This model is in fact more complex since the PKA is able to phosphorylate the Msn2 protein on both the nuclear localization signal (NLS) and the nuclear export signal (NES) of this protein, whereas PP2A only dephosphorylates NES (Gorner et al. 2002; Santhanam et al. 2004), indicating a prominent effect of the PKA in controlling the localization of Msn2/4p and consequently on Msn2-dependent gene expression. Nonetheless, the mechanism by which nitrogen starvation, heat, or osmotic shock promote activation

of glycogen and trehalose-related genes is mainly due to the PP2A-dependent dephosphorylation as there is no evidence that the PKA activity is modified under these conditions (Zaman et al. 2008) (Fig. 4). However, this model cannot account for the independency between the TOR and the PKA in glycogen accumulation as shown by the fact that yeast cells bearing unbridled PKA activity (i.e. *bcy1* mutant) and treated with rapamycin are still able to accumulate glycogen (Barbet et al. 1996; Zurita-Martinez and Cardenas2005). A possible explanation for this effect is to propose that the apparent glycogen accumulation is actually a consequence of the sequestration of this glucose polymer into the vacuole by the rapamycin-dependent induction of the autophagy process (Noda and Ohsumi 1998; Dubouloz et al. 2005), implying inhibition of the TORC1 but being independent to Msn2/4p and Rim15p (Budovskaya et al. 2004; Yorimitsu et al. 2007) (Fig. 4).

Two additional nutrient-sensor kinases, namely the cyclin-dependent Pho85 kinase and the Snf1 kinase, have been reported to control glycogen and trehalose. However, contrary to the PKA and the TORC1 pathways, which merely exert control at the transcriptional level (see Fig. 4), the regulation by Snf1p and Pho85p takes place at both transcriptional and posttranslational levels. A two- to threefold upregulation or downregulation of glycogen and trehalose metabolism-related genes has been reported in *pho85* and *snf1* mutants, respectively (Timblin and Bergman 1997; Parrou et al. 1999b), but the mechanisms of this control are not yet determined. The presence of binding sites for the transcriptional factors Adr1p or Mig1p in most of glycogen and trehalose-related genes could be the mechanism through which Snf1 kinase exerts its positive transcriptional effect (Fig. 4), whereas effects of Pho85 kinase on expression of these genes are still unclear (Enjalbert et al. 2004). At the posttranslational level, the positive control on glycogen accumulation by Snf1p appears to involve two distinct pathways. On the one hand, the Snf1 kinase has been shown to antagonize the Pho85-dependent phosphorylation of Gsy2p (Huang et al. 1996; Wilson et al. 1999), but how this antagonism takes place is not yet understood. On the other hand, Snf1p can indirectly affect glycogen store through its positive control of the autophagy process involving *APG1* and *APG13* (Wang et al. 2001), as this latter mechanism causes part of glycogen particles to be stored into the vacuole. This process being defective in a *snf1* mutant would account in part for the lack of glycogen in this mutant. Finally, the recovery of glycogen in an *snf1pho85* mutant is explained by a concomitant hyperactivation of glycogen synthase and an apparent recovery of the autophagy process, indicating that Pho85 also controls in an antagonistic manner to Snf1 the autophagy process (Wang et al. 2001).

In summary, several nutrient-sensing pathways impinge on glycogen and trehalose metabolic systems at the transcriptional and posttranslational levels. The PKA pathway is clearly the major transcriptional mechanism of control, whereas Glc6P is the major metabolic effector as it is a direct substrate for trehalose synthesis, a potent activator of glycogen synthase and an inhibitor of glycogen phosphorylase, and last but not least, its binding to these two enzymes favors the dephosphorylation and inhibits the phosphorylation processes (Fig. 4) (Francois and Parrou 2001). Under efficient growth related to available nutrients, such as during growth on glucose rich medium, glycogen and trehalose accumulation is prevented because

the PKA and the TORC1 are fully operative, whereas under growth imbalance related with some nutrients shortage, accumulation of these two glucose stores may be favored. However, the nature of the growth-limiting nutrient is critical for effective accumulation of glycogen and trehalose. In excess of glucose in a nitrogen-depleted medium, the high Glc6P prevailing in this condition favors synthesis of glycogen and trehalose (Francois et al. 1988; Parrou et al. 1999b; Hazelwood et al. 2009). On the other hand, limitation or depletion of sulfate, phosphate, or zinc is not accompanied by the rise of glycogen or trehalose because under this growth-limiting condition, the PKA and the TOR pathways are activated, as indicated by low transcript levels of glycogen and trehalose-related genes (Hazelwood et al. 2009). Finally, it is noteworthy that the coexpression of genes in the biosynthetic and the biodegradation pathways mainly due to the presence of STRE in their promoter may lead to a recycling of trehalose and glycogen (Blomberg 2000; Voit 2003). Whether this recycling, which has been genetically demonstrated to exist under heat shock, saline stresses, and during growth on glucose (Parrou et al. 1997; Parrou et al. 1999b; Pedreno et al. 2002; Mahmud et al. 2009), has any physiological meaning or is a fortuitous consequence of the coexpression of these genes remains to be addressed.

4 Biological Function of Storage Carbohydrates in Yeast

It is well established that glycogen and trehalose are two energy stores for the yeast cells. This section discusses more precisely how and when yeast cells are playing with these two glucose stores thanks to the use of more sophisticated bioprocess conditions combined with the use of dedicated mutants. Besides, the trehalose synthesis pathways are endowed with a peculiar function that is likely needed in the regulation of the energy and carbon metabolism in yeast.

4.1 Function as Energy and Carbon Stores

Glycogen agrees with the concept of an energy store since it is found to accumulate when glucose is still present in the medium, and is only mobilized when all exogenous carbon sources have been exhausted (Parrou et al. 1999b; Wang et al. 2001). Trehalose does not exactly fit with this concept since it accumulates only after glucose has been consumed (Francois et al. 1991). Nevertheless, several biological situations indicate that both glucose stores have an energetic function in yeast cells. A relevant example is found with respiratory-deficient mutant cells, which accumulate larger amount of glycogen during the growth phase on glucose and then readily mobilize it at the onset of glucose depletion because these cells are respiratory deficient and hence cannot resume on the accumulated ethanol or amino acids present in the growth medium. This rapid mobilization coincides with a drop of Glc6P, accompanied by an increase of glycogen phosphorylase and decrease of

glycogen synthase activity (Enjalbert et al. 2000) by a mechanism that may implicate Pho85p kinase as well as other uncharacterized partners (Wilson et al. 2002a). It is worth noting that respiratory mutants are also unable to accumulate trehalose (J. François, unpublished data). A seemingly direct function of trehalose in carbon and energy metabolism has been recently underscored from studies aiming at characterizing whether Ath1 can hydrolyze endogenous trehalose. In this work, an *nth1nth2* mutant defective in cytosolic trehalases was grown on trehalose and then subjected to carbon starvation. This extreme situation resulted in a rapid mobilization of trehalose by a mechanism involving first its export out of the cell by a yet uncharacterized exporter, the hydrolysis of the exported trehalose at the cell surface by Ath1p, and the subsequent uptake of the released glucose (Jules et al. 2008).

It is well known that accumulation of reserve carbohydrates is favored at lower growth rates under carbon- or nitrogen-limited conditions. In fact, this accumulation is proportional to the duration of the G1 phase of the growth cycle (Sillje et al. 1999; Paalman et al. 2003) and correlates with the transcriptional activation of glycogen and trehalose-related genes (Brauer et al. 2008; Hazelwood et al. 2009). In contrast, it is reduced by overexpression of the G1 cyclin Cln3, the translation rate of which is positively regulated by the TORC1 kinase (Barbet et al. 1996). These data are in accordance with the recent proposition that TORC1 is the major controller of growth rate in response to nutrient availability (Castrillo et al. 2007). The stored carbohydrates can be readily mobilized upon raising the growth rate, and this rapid mobilization is likely to supply ATP surplus required for budding process since a good correlation has been obtained between the increase in the budding index and the extension of reserve carbohydrates mobilization, when the growth rate was suddenly increased from 0.05 to 0.15 h^{-1} (Guillou et al. 2004). This experimental approach is strongly reminiscent to the energy-metabolism oscillations (EMO) that arise spontaneously under glucose- or nitrogen-limited continuous cultures at low dilution (growth) rate, showing periodicity of approximately 300 min of waves of accumulation and mobilization of reserve carbohydrates, as first reported almost 40 years ago (Kuenzi and Fiechter 1972). Such oscillatory behavior has been also observed in batch culture of yeast on trehalose (Jules et al. 2005). This EMO has been recently investigated in a system-level approach, showing that it is composed of two distinct phases termed respiro-fermentative and respiratory period, respectively. The transition between these two periods is basically characterized by a periodic change in the $NADH/NAD^+$ ratio, where the ratio is high during the respiro-fermentative period and low during the respiratory period (Xu and Tsurugi 2006). The importance of trehalose and glycogen in controlling EMO has been illustrated using mutants defective in the synthesis of trehalose (*tps1* mutant) that exhibit destabilized EMO (Xu and Tsurugi 2007), while mutants defective in glycogen (*gsy2* or *gsy2gsy1p* mutants) show very weak oscillatory waves (Xu and Tsurugi 2006; J. Francois, unpublished results). At a global transcriptomic level, these spontaneous oscillations, which were also termed yeast metabolic cycle (YMC), revealed that over half of yeast genes exhibited periodic expression patterns, with a common period of transcript oscillation of ~300 min (Tu et al. 2005). Using an

unbiased k-means cluster analysis, these authors identified three superclusters defining a temporal compartmentalization of the oscillations in three major phases, namely the Ox (oxidative), the R/B (reductive-building), and R/C (reductive-charging) phase, respectively. The Ox clusters mainly comprises genes involved in ribosome and protein synthesis, the R/B cluster was enriched of genes encoding proteins required for DNA replication and genes encoding mitochondrial proteins, whereas the R/C supercluster contained proteins involved in protein degradation, peroxisomes, fatty acid oxidation as well as genes of the glycogen and trehalose metabolism. Taking into account the metabolic events identified in the EMO, the respiratory phase would correspond to the last part of R/B and R/C, as it is the period during which storage carbohydrate accumulates and the respiratory quotient (RQ) is close to 1.0. On the other hand, the respiro-fermentative phase corresponds to Ox and to the beginning of R/B during which stored carbohydrates are liquidated and the RQ $>$ 1.0, corresponding to a reductive, highly glycolytic metabolism. Recently, an interesting model was proposed that the temporal compartmentalization of respiration and the restriction of DNA replication to the reductive phase of the metabolic cycle are to protect cells for genomic integrity (Chen et al. 2007). However, another model assigns the sudden mobilization of reserve carbohydrates to specific metabolic requirements to pass the START at the G1/S transition of the cell cycle, as proposed by Futcher (Futcher 2006). This author proposed the "finishing kick" hypothesis which states that at low growth rate, the cell organizes its metabolism to store sufficient carbohydrates during the G1 phase then suddenly burns it to provide an additional burst of ATP for biosynthesis processes in late G1, resulting in increased budding rate. This finishing kick hypothesis also suggests that the critical size that has to be reached to pass through the Start could be correlated to the stored carbohydrates. However, the function of reserve carbohydrate as cell sizer remains to be verified. Moreover, the hypothesis of a finishing kick is only valid for slow growing cells since rapidly growing cells do not store glycogen or trehalose but show normal cell cycle progression.

While the mechanism that governs the synthesis of glycogen and trehalose during G1 may be dependent on a reduction of the TORC1 activity, the rapid mobilization of the stored carbohydrates in late G1 coincided with a transient burst of cAMP (Xu and Tsurugi 2006; Muller et al. 2003). This suggests that mobilization of the stored carbohydrate is mediated by the PKA pathway. In favor of this model, trehalase and glycogen phosphorylase activity was found to increase at this period (Muller et al. 2003; J. François, unpublished data).

4.2 Specific Function of Trehalose as a Stress Protectant

A number of reports have shown that the trehalose molecule is endowed with the unique property to act as a replacement of water molecule to stabilize proteins and membranes from dessication. However, recent results indicate that trehalose is neither necessary nor sufficient for dessication tolerance in yeast (Ratnakumar and

Tunnacliffe 2006). Thermotolerance has also been reported to be a synergistic effect due to the accumulation of trehalose acting as chemical chaperones and molecular chaperones (Singer and Lindquist 1998; Lee and Goldberg 1998). However, Thevelein's group recently showed that additional unidentified factors may participate in this resistance since a mutant strain defective in adenylate cyclase (*fil1* mutant) that was rendered unable to accumulate trehalose and lacking also Hsp104 protein still exhibited elevated thermotolerance (Versele et al. 2004). Besides, the role of trehalose in the acquisition of thermotolerance may be dependent on the property of this disaccharide to activate Hsf1 (Bulman and Nelson 2005; Conlin and Nelson 2007), which is an essential transcriptional regulator of heat shock response in eukaryote (Amoros and Estruch 2001). On the other hand, recovery of viability of cells from heat shock or saline stress required that the accumulated trehalose is mobilized, to allow proper recovery to normal conditions (Wera et al. 1999; Garre and Matallana 2009). The proposed explanation is that the disaccharide can interfere with the refolding of denatured proteins by HSPs that takes place upon return from heat shock or salt stress (Singer and Lindquist 1998). The adaptation of yeast cells to near-freezing temperatures seems also to be linked to the presence of trehalose. Shifting temperature from 25 to $< 10°C$ is accompanied by a dramatic rise in trehalose and by a Msn2/Msn4p-dependent induction of genes related to its synthesis as well as genes encoding some HSP proteins (Panadero et al. 2006; Schade et al. 2004). It has been observed that an *msn2*/*msn4* mutant dies quickly when maintained at temperature below 10°C (Kandror et al. 2004), but it has not been shown whether this rapid death was due to the lack of trehalose. Finally, trehalose protects cells from damage induced by oxygen radicals as well as from ethanol toxicity (Benaroudj et al. 2001; van Voorst et al. 2006). These protective effects are likely due to the property of this disaccharide to prevent proteins to be damaged under these harsher conditions.

5 The Role of Tps1/ Trehalose-6-Phosphate in Carbon and Energy Metabolism

An unexpected link between the trehalose and the glycolytic pathway is that mutations in *TPS1* prevent growth on rapidly fermentable carbon sources (reviewed in (Gancedo and Flores 2004). The metabolic phenotype that characterizes this mutant is a massive accumulation of sugar phosphates and precipitous depletion of ATP immediately after glucose addition. These effects are likely responsible for the inability of a *tps1* mutant to growth on glucose. The *tps1* mutant can grow on less rapidly fermented sugars such as galactose or raffinose that also depend on the function of the glycolytic pathway causing, however, lower flux over this pathway. In addition, catabolism of these sugars differs from the one of glucose in the sensing and the uptake mechanisms, respectively (Gancedo 2008). This led to the hypothesis that the lack of growth of the *tps1* mutant could be caused by deregulation of the transport step or of the glucose phosphorylating activity.

Three models have been put forward trying to explain the involvement of Tps1 in the control of the sugar influx and, by extension, in the regulation of glycolysis. The first model is based on the finding that the main hexokinase in yeast encoded by *HXK2* is inhibited by Tre6P, the product of the Tps1p reaction (Blazquez et al. 1993). Though quite attractive at first sight, the Tre6P inhibition model of hexokinase is probably incomplete since yeast cells growing exponentially on glucose (and thus in a highly glycolytic state) or overexpressing *TPS2* that encodes the trehalose 6-P phosphatase have barely detectable Tre6P levels while growth on glucose is not impaired (Hohmann et al. 1996). Also, growth and fermentative capacity of yeast are not altered after replacement of a Tre6P-sensitive hexokinase by an enzyme insensitive to this metabolite (Bonini et al. 2003). More importantly, it was recently found that *TPS1* from *Y. lypolitica* fully complemented growth of an *S. cerevisiae tps1Δ* mutant on fructose, even though Tre6P was barely detected in this mutant (C. Gancedo and J. François, unpublished results). Such a result brings us to the second hypothesis which proposes that besides its catalytic function, Tps1p may have a regulatory role, as for instance by restricting sugar influx through a yet unidentified protein interaction. Evidence in support of this hypothesis came from work on the pathogenic fungus *Magnaporthe grisea*, in which the introduction of a noncatalytic form of Tps1p into *tps1* mutants from this fungus recovered its capacity to invade rice leaves which was lost upon deletion of the protein. Another interesting observation was made in the model plant *A. thaliana* where a single point mutation in the *AtTPS6* gene resulted in many phenotypes, although the mutated variant protein kept its catalytic function (Chary et al. 2008). In yeast, there is so far no direct evidence supporting the hypothesis of a regulatory role exerted by the Tps1 protein independently from its reaction product, Tre6P. However, the fact that *tps1* mutants are also unable to undergo sporulation, a process that occurs in the absence of fermentable carbon sources and thus independently from Tre6P, supports the idea that the Tps1 protein has functions other than the simple formation of the Tre6P from UDP-Glc and Glc6P (Silva-Udawatta and Cannon 2001). In addition, it was shown that Tps1p may be present as a free protein (i.e., not bound to the TPS protein complex) (Bell et al. 1998), and recent global interactomics studies indicate that Tps1p may belong to a large interactomic network, whose partners mainly fall into the MIPS categories of energy and metabolism (27%), cell rescue and defense (12%), and cell cycle and DNA processing (10%) (Krogan et al. 2006; Gavin et al. 2006). The third hypothesis proposes that the trehalose biosynthetic pathway can serve an additional function, i.e., in the recovery of inorganic phosphate that is required for the functioning of glycolysis at the level of glyceraldehyde 3-P dehydrogenase. The importance of Pi replenishment in rescuing growth of *tps1* on highly fermentative sugars has been illustrated by hyperactivity of the Gpd1p and/or of the glycerol facilitator encoded by *FPS1* (Van Aelst et al. 1991) that both result in excess glycerol formation at the expense of triose intermediates DHAP. The rapid drop Pi in a *tps1* mutant is likely not collateral effect of the lack of sugar influx but may be a direct consequence of the lack of activation of H^+-ATPase in a *tps1* mutant (Th Walther and J François, unpublished results).

Recent genome-wide analyses on pairwise genetic interactions have provided new insights on how Tps1p may impinge on cellular growth. These results indicated that *TPS1* negatively interacts with more than 200 genes, whose functions mainly fall into the MIPS functional categories vesicle formation and vesicular transport, phosphate metabolism, budding/cell polarity, cell wall, and general stress response. It suggests that the levels of Tre6P or the Tps1 protein itself may be critical in regulating some targeted cellular functions by coordinating sugar metabolism with cell growth, budding, and cell wall synthesis according to carbon availability. A similar hypothesis has been raised for the role of Tre6P in plant in coordinating sugar metabolism with development, particularly with the cell wall synthesis that depends on the supply of Glc6P and UDP-Glc (Paul et al. 2008). Also and quite intriguingly, a considerable number of genes that negatively interact with *TPS1* were found to positively interact with *TPS2* and *vice versa* (Fiedler et al. 2009; Costanzo et al. 2010). These genes provide candidate cellular functions that are controlled by Tre6P since this metabolite is absent in *tps1* mutants and exhibits hyperaccumulation in *tps2* strains. Genes that show negative interaction with *TPS1* and positive interaction with *TPS2* include (among others) *ANP1, RIM20, CHS5, PSD1, WHI2, COG7, RSP5, DFG16, ADO1, ATX1,* and *VSP9* , whereas the opposite situation is found for *HXK2, UBR1, PAP1, PMA1, YHC1*, and *CAK1*. These findings support a potential *direct* implication of Tre6P in the regulation of vesicle formation, phospholipid metabolism, and Pi/ATP homeostasis. In short, converging data strongly support an essential function of Tps1 and its metabolite Tre6P in the regulation of carbon and energy metabolism in yeast, for which the precise mechanism of the action and relevant cellular targets remain to be identified.

6 Conclusion and Perspectives

The yeast *S. cerevisiae* accumulates two storage carbohydrates, glycogen and trehalose, that fulfill and share, in some cases, specific functions. The control of the metabolism of these two glucose stores is extremely sophisticated and is likely meant to satisfy rapidly changing energetic needs during cell cycle and upon changes in nutrient availability. Among numerous questions regarding mechanisms by which the nutrient-sensing pathways impact on storage carbohydrate metabolism, two major problems need to be solved. The first one is to identify the alternative priming system that allows glycogen to be synthesized without glycogenin that also includes the elucidation of the stochastic nature of this alternative mechanism. Answering this question can be expected to have a strong impact on human glycogen and its related metabolic disorders. The second question is to unravel the complete mechanism by which the TPS complex and/or Tre6P regulate glycolysis and energy metabolism. It is essential to address this question because it is indispensable for our general understanding of fermentative growth. It is also a necessary step toward rational engineering of the glycolytic pathway, being it dedicated to improve fermentation of

natural substrates or to enable fermentation of nonnaturally consumed carbon sources like xylose.

Acknowledgments We are grateful to many colleagues for stimulating discussion and for providing unpublished data. This study was supported by EU grants (4th and 5th Frameworks), Genopole Toulouse, Agence Nationale de la Recherche (ANR contract NT05-2_42127), and Bonus Quality Research-INSA 2009–2010.

References

Alonso MD, Lagzdins EJ, Lomako J, Lomako WM, Whelan WJ (1995a) New and specific nucleoside diphosphate glucose substrates for glycogenin. FEBS Lett 359:110–112

Alonso MD, Lomako J, Lomako WM, Whelan WJ (1995b) A new look at the biogenesis of glycogen. FASEB J 9:1126–1137

Amoros M, Estruch F (2001) Hsf1p and Msn2/4p cooperate in the expression of Saccharomyces cerevisiae genes HSP26 and HSP104 in a gene- and stress type-dependent manner. Mol Microbiol 39:1523–1532

App H, Holzer H (1989) Purification and characterization of neutral trehalase from the yeast ABYS1 mutant. J Biol Chem 264:17583–17588

Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006) Insights on the evolution of trehalose biosynthesis. BMC Evol Biol 6:109

Avonce N, Wuyts J, Verschooten K, Vandesteene L, Van Dijck P (2010) The Cytophaga hutchinsonii ChTPSP: first characterized bifunctional TPS-TPP protein as putative ancestor of all eukaryotic trehalose biosynthesis proteins. Mol Biol Evol 27:359–369

Barbet NC, Schneider U, Helliwell SB, Stansfield I, Tuite MF, Hall MN (1996) TOR controls translation initiation and early G1 progression in yeast. Mol Biol Cell 7:25–42

Baskaran S, Roach PJ, DePaoli-Roach AA, Hurley TD (2010) Structural basis for glucose-6-phosphate activation of glycogen synthase. Proc Natl Acad Sci USA 107:17563–17568

Bell W, Sun W, Hohmann S, Wera S, Reinders A, De Virgilio C, Wiemken A, Thevelein JM (1998) Composition and functional analysis of the Saccharomyces cerevisiae trehalose synthase complex. J Biol Chem 273:33311–33319

Benaroudj N, Lee DH, Goldberg AL (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. J Biol Chem 276:24261–24267

Blazquez MA, Lagunas R, Gancedo C, Gancedo JM (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Lett 329:51–54

Blomberg A (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett 182:1–8

Bonini BM, Van Dijck P, Thevelein JM (2003) Uncoupling of the glucose growth defect and the deregulation of glycolysis in *Saccharomyces cerevisiae* Tps1 mutants expressing trehalose-6-phosphate-insensitive hexokinase from *Schizosaccharomyces pombe*. Biochim Biophys Acta 1606:83–93

Brauer MJ, Huttenhower C, Airoldi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D (2008) Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. Mol Biol Cell 19:352–367

Budovskaya YV, Stephan JS, Reggiori F, Klionsky DJ, Herman PK (2004) The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in *Saccharomyces cerevisiae*. J Biol Chem 279:20663–20671

Bulman AL, Nelson HC (2005) Role of trehalose and heat in the structure of the C-terminal activation domain of the heat shock transcription factor. Proteins 58:826–835

Castrillo JI, Zeef LA, Hoyle DC, Zhang N, Hayes A, Gardner DC, Cornell MJ, Petty J, Hakes L, Wardleworth L, Rash B, Brown M, Dunn WB, Broadhurst D, O'Donoghue K, Hester SS, Dunkley TP, Hart SR, Swainston N, Li P, Gaskell SJ, Paton NW, Lilley KS, Kell DB, Oliver SG (2007) Growth control of the eukaryote cell: a systems biology study in yeast. J Biol 6:4

Chary SN, Hicks GR, Choi YG, Carter D, Raikhel NV (2008) Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol 146:97–107

Chen Z, Odstrcil EA, Tu BP, McKnight SL (2007) Restriction of DNA replication to the reductive phase of the metabolic cycle protects genome integrity. Science 316:1916–1919

Cheng C, Mu J, Farkas I, Huang D, Goebl MG, Roach PJ (1995) Requirement of the self-glucosylating initiator proteins Glg1p and Glg2p for glycogen accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 15:6632–6640

Cheng C, Huang D, Roach PJ (1997) Yeast *PIG* genes: *PIG1* encodes a putative type 1 phosphatase subunit that interacts with the yeast glycogen synthase Gsy2p. Yeast 13:1–8

Chu S, DeRisi J, Eisen M, Mulholland J, Botstein D, Brown PO, Herskowitz I (1998) The transcriptional program of sporulation in budding yeast. Science 282:699–705

Clancy MJ, Smith LM, Magee PT (1982) Developmental regulation of a sporulation-specific enzyme activity in *Saccharomyces cerevisiae*. Mol Cell Biol 2:171–178

Clotet J, Posas F, Hu GZ, Ronne H, Arino J (1995) Role of protein phosphatase 2A in the control of glycogen metabolism in yeast. Eur J Biochem 229:207–214

Colonna WJ, Magee PT (1978) Glycogenolytic enzymes in sporulating yeast. J Bacteriol 134:844–853

Conlin LK, Nelson HC (2007) The natural osmolyte trehalose is a positive regulator of the heat-induced activity of yeast heat shock transcription factor. Mol Cell Biol 27:1505–1515

Corominas J, Clotet J, Fernandez BI, Boles E, Zimmermann FK, Guinovart JJ, Arino J (1992) Glycogen metabolism in a *Saccharomyces cerevisiae* phosphoglucose isomerase (*pgil*) disruption mutant. FEBS Lett 310:182–186

Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS, Ding H, Koh JL, Toufighi K, Mostafavi S, Prinz J, St Onge RP, VanderSluis B, Makhnevych T, Vizeacoumar FJ, Alizadeh S, Bahr S, Brost RL, Chen Y, Cokol M, Deshpande R, Li Z, Lin ZY, Liang W, Marback M, Paw J, San Luis BJ, Shuteriqi E, Tong AH, van Dyk N, Wallace IM, Whitney JA, Weirauch MT, Zhong G, Zhu H, Houry WA, Brudno M, Ragibizadeh S, Papp B, Pal C, Roth FP, Giaever G, Nislow C, Troyanskaya OG, Bussey H, Bader GD, Gingras AC, Morris QD, Kim PM, Kaiser CA, Myers CL, Andrews BJ, Boone C (2010) The genetic landscape of a cell. Science 327:425–431

Daran JM, Dallies N, Thines-Sempoux D, Paquet V, Francois J (1995) Genetic and biochemical characterization of the *UGP1* gene encoding the UDP-glucose pyrophosphorylase from *Saccharomyces cerevisiae*. Eur J Biochem 233:520–530

Daran JM, Bell W, Francois J (1997) Physiological and morphological effects of genetic alterations leading to a reduced synthesis of UDP-glucose in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 153:89–96

De Virgilio C, Loewith R (2006) The TOR signalling network from yeast to man. Int J Biochem Cell Biol 38:1476–1481

Dubouloz F, Deloche O, Wanke V, Cameroni E, De Virgilio C (2005) The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol Cell 19:15–26

Elliott B, Haltiwanger RS, Futcher B (1996) Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. Genetics 144:923–933

Enjalbert B, Parrou JL, Vincent O, Francois J (2000) Mitochondrial respiratory mutants of *Saccharomyces cerevisiae* accumulate glycogen and readily mobilize it in a glucose-depleted medium. Microbiology 146(Pt 10):2685–2694

Enjalbert B, Parrou JL, Teste MA, Francois J (2004) Combinatorial control by the protein kinases PKA, PHO85 and SNF1 of transcriptional induction of the *Saccharomyces cerevisiae* GSY2 gene at the diauxic shift. Mol Genet Genomics 271:697–708

Farkas I, Hardy TA, DePaoli-Roach AA, Roach PJ (1990) Isolation of the *GSY1* gene encoding yeast glycogen synthase and evidence for the existence of a second gene. J Biol Chem 265:20879–20886

Farkas I, Hardy TA, Goebl MG, Roach PJ (1991) Two glycogen synthase isoforms in *Saccharomyces cerevisiae* are coded by distinct genes that are differentially controlled. J Biol Chem 266:15602–15607

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat Biotechnol 20:301–305

Fiedler D, Braberg H, Mehta M, Chechik G, Cagney G, Mukherjee P, Silva AC, Shales M, Collins SR, van Wageningen S, Kemmeren P, Holstege FC, Weissman JS, Keogh MC, Koller D, Shokat KM, Krogan NJ (2009) Functional organization of the *S. cerevisiae* phosphorylation network. Cell 136:952–963

Fletterick RJ, Burke JA, Hwang PK, Nakano K, Newgard CB (1986) Structural relationships in glycogen phosphorylases. Ann N Y Acad Sci 478:220–232

Francois J, Hers HG (1988) The control of glycogen metabolism in yeast. 2. A kinetic study of the two forms of glycogen synthase and of glycogen phosphorylase and an investigation of their interconversion in a cell-free extract. Eur J Biochem 174:561–567

Francois J, Parrou JL (2001) Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:125–145

Francois J, Villanueva ME, Hers HG (1988) The control of glycogen metabolism in yeast. 1. Interconversion in vivo of glycogen synthase and glycogen phosphorylase induced by glucose, a nitrogen source or uncouplers. Eur J Biochem 174:551–559

Francois J, Neves MJ, Hers HG (1991) The control of trehalose biosynthesis in Saccharomyces cerevisiae: evidence for a catabolite inactivation and repression of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. Yeast 7:575–587

Francois JM, Thompson-Jaeger S, Skroch J, Zellenka U, Spevak W, Tatchell K (1992) GAC1 may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. EMBO J 11:87–96

Fukuhara H (2003) The Kluyver effect revisited. FEMS Yeast Res 3:327–331

Futcher B (2006) Metabolic cycle, cell cycle, and the finishing kick to start. Genome Biol 7:107

Gancedo JM (2008) The early steps of glucose signalling in yeast. FEMS Microbiol Rev 32:673–704

Gancedo C, Flores CL (2004) The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Res 4:351–359

Garre E, Matallana E (2009) The three trehalases Nth1p, Nth2p and Ath1p participate in the mobilization of intracellular trehalose required for recovery from saline stress in *Saccharomyces cerevisiae*. Microbiology 155:3092–3099

Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, Marzioch M, Rau C, Jensen LJ, Bastuck S, Dumpelfeld B, Edelmann A, Heurtier MA, Hoffman V, Hoefert C, Klein K, Hudak M, Michon AM, Schelder M, Schirle M, Remor M, Rudi T, Hooper S, Bauer A, Bouwmeester T, Casari G, Drewes G, Neubauer G, Rick JM, Kuster B, Bork P, Russell RB, Superti-Furga G (2006) Proteome survey reveals modularity of the yeast cell machinery. Nature 440:631–636

Gorner W, Durchschlag E, Wolf J, Brown EL, Ammerer G, Ruis H, Schuller C (2002) Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. EMBO J 21:135–144

Guillou V, Plourde-Owobi L, Parrou JL, Goma G, Francois J (2004) Role of reserve carbohydrates in the growth dynamics of *Saccharomyces cerevisiae*. FEMS Yeast Res 4:773–787

Han EK, Cotty F, Sottas C, Jiang H, Michels CA (1995) Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. Mol Microbiol 17:1093–1107

Hardy TA, Roach PJ (1993) Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. J Biol Chem 268:23799–23805

Hazelwood LA, Walsh MC, Luttik MA, Daran-Lapujade P, Pronk JT, Daran JM (2009) Identity of the growth-limiting nutrient strongly affects storage carbohydrate accumulation in anaerobic chemostat cultures of Saccharomyces cerevisiae. Appl Environ Microbiol 75:6876–6885

He S, Bystricky K, Leon S, Francois JM, Parrou JL (2009) The Saccharomyces cerevisiae vacuolar acid trehalase is targeted at the cell surface for its physiological function. FEBS J 276:5432–5446

Hohmann S, Bell W, Neves MJ, Valckx D, Thevelein JM (1996) Evidence for trehalose-6-phosphate-dependent and -independent mechanisms in the control of sugar influx into yeast glycolysis. Mol Microbiol 20:981–991

Huang D, Farkas I, Roach PJ (1996) Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 16:4357–4365

Huang D, Wilson WA, Roach PJ (1997) Glucose-6-P control of glycogen synthase phosphorylation in yeast. J Biol Chem 272:22495–22501

Huang D, Moffat J, Wilson WA, Moore L, Cheng C, Roach PJ, Andrews B (1998) Cyclin partners determine Pho85 protein kinase substrate specificity in vitro and in vivo: control of glycogen biosynthesis by Pcl8 and Pcl10. Mol Cell Biol 18:3289–3299

Huang J, Reggiori F, Klionsky DJ (2007) The transmembrane domain of acid trehalase mediates ubiquitin-independent multivesicular body pathway sorting. Mol Biol Cell 18:2511–2524

Hwang PK, Tugendreich S, Fletterick RJ (1989) Molecular analysis of *GPH1*, the gene encoding glycogen phosphorylase in *Saccharomyces cerevisiae*. Mol Cell Biol 9:1659–1666

Jules M, Guillou V, Francois J, Parrou JL (2004) Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:2771–2778

Jules M, Francois J, Parrou JL (2005) Autonomous oscillations in *Saccharomyces cerevisiae* during batch cultures on trehalose. FEBS J 272:1490–1500

Jules M, Beltran G, Francois J, Parrou JL (2008) New insights into trehalose metabolism by *Saccharomyces cerevisiae*: NTH2 encodes a functional cytosolic trehalase, and deletion of TPS1 reveals Ath1p-dependent trehalose mobilization. Appl Environ Microbiol 74:605–614

Kandror O, Bretschneider N, Kreydin E, Cavalieri D, Goldberg AL (2004) Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. Mol Cell 13:771–781

Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, Punna T, Peregrin-Alvarez JM, Shales M, Zhang X, Davey M, Robinson MD, Paccanaro A, Bray JE, Sheung A, Beattie B, Richards DP, Canadien V, Lalev A, Mena F, Wong P, Starostine A, Canete MM, Vlasblom J, Wu S, Orsi C, Collins SR, Chandran S, Haw R, Rilstone JJ, Gandi K, Thompson NJ, Musso G, St Onge P, Ghanny S, Lam MH, Butland G, Altaf-Ul AM, Kanaya S, Shilatifard A, O'Shea E, Weissman JS, Ingles CJ, Hughes TR, Parkinson J, Gerstein M, Wodak SJ, Emili A, Greenblatt JF (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature 440:637–643

Kuenzi MT, Fiechter A (1972) Regulation of carbohydrate composition of *Saccharomyces cerevisiae* under growth limitation. Arch Microbiol 84:254–265

Kwon HB, Yeo ET, Hahn SE, Bae SC, Kim DY, Byun MO (2003) Cloning and characterization of genes encoding trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) from Zygosaccharomyces rouxii. FEMS Yeast Res 3:433–440

Lee DH, Goldberg AL (1998) Proteasome inhibitors cause induction of heat shock proteins and trehalose, which together confer thermotolerance in *Saccharomyces cerevisiae*. Mol Cell Biol 18:30–38

Lin K, Hwang PK, Fletterick RJ (1995) Mechanism of regulation in yeast glycogen phosphorylase. J Biol Chem 270:26833–26839

Lin K, Rath VL, Dai SC, Fletterick RJ, Hwang PK (1996) A protein phosphorylation switch at the conserved allosteric site in GP. Science 273:1539–1542

Lomako J, Lomako WM, Whelan WJ (2004) Glycogenin: the primer for mammalian and yeast glycogen synthesis. Biochim Biophys Acta 1673:45–55

Londesborough J, Varimo K (1984) Characterization of two trehalases in baker's yeast. Biochem J 219:511–518

Londesborough J, Vuorio OE (1993) Purification of trehalose synthase from baker's yeast. Its temperature-dependent activation by fructose 6-phosphate and inhibition by phosphate. Eur J Biochem 216:841–848

Mahmud SA, Nagahisa K, Hirasawa T, Yoshikawa K, Ashitani K, Shimizu H (2009) Effect of trehalose accumulation on response to saline stress in *Saccharomyces cerevisiae*. Yeast 26:17–30

Melendez R, Melendez-Hevia E, Canela EI (1999) The fractal structure of glycogen: a clever solution to optimize cell metabolism. Biophys J 77:1327–1332

Muller D, Exler S, Aguilera-Vazquez L, Guerrero-Martin E, Reuss M (2003) Cyclic AMP mediates the cell cycle dynamics of energy metabolism in *Saccharomyces cerevisiae*. Yeast 20:351–367

Neves MJ, Jorge JA, Francois JM, Terenzi HF (1991) Effects of heat shock on the level of trehalose and glycogen, and on the induction of thermotolerance in *Neurospora crassa*. FEBS Lett 283:19–22

Ni HT, LaPorte DC (1995) Response of a yeast glycogen synthase gene to stress. Mol Microbiol 16:1197–1205

Noda T, Ohsumi Y (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 273:3963–3966

Nwaka S, Mechler B, Destruelle M, Holzer H (1995) Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*. FEBS Lett 360:286–290

Nwaka S, Mechler B, Holzer H (1996) Deletion of the ATH1 gene in *Saccharomyces cerevisiae* prevents growth on trehalose. FEBS Lett 386:235–238

Paalman JW, Verwaal R, Slofstra SH, Verkleij AJ, Boonstra J, Verrips CT (2003) Trehalose and glycogen accumulation is related to the duration of the G1 phase of *Saccharomyces cerevisiae*. FEMS Yeast Res 3:261–268

Panadero J, Pallotti C, Rodriguez-Vargas S, Randez-Gil F, Prieto JA (2006) A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. J Biol Chem 281:4638–4645

Panni S, Landgraf C, Volkmer-Engert R, Cesareni G, Castagnoli L (2008) Role of 14-3-3 proteins in the regulation of neutral trehalase in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 8:53–63

Parrou JL, Teste MA, François J (1997) Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology 143:1891–1900

Parrou JL, Enjalbert B, Francois J (1999a) STRE- and cAMP-independent transcriptional induction of *Saccharomyces cerevisiae GSY2* encoding glycogen synthase during diauxic growth on glucose. Yeast 15:1471–1484

Parrou JL, Enjalbert B, Plourde L, Bauche A, Gonzalez B, Francois J (1999b) Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. Yeast 15:191–203

Parrou JL, Jules M, Beltran G, Francois J (2005) Acid trehalase in yeasts and filamentous fungi: localization, regulation and physiological function. FEMS Yeast Res 5:503–511

Paul MJ, Primavesi LF, Jhurreea D, Zhang Y (2008) Trehalose metabolism and signaling. Annu Rev Plant Biol 59:417–441

Pederson BA, Cheng C, Wilson WA, Roach PJ (2000) Regulation of glycogen synthase. Identification of residues involved in regulation by the allosteric ligand glucose-6-P and by phosphorylation. J Biol Chem 275:27753–27761

Pederson BA, Wilson WA, Roach PJ (2004) Glycogen synthase sensitivity to glucose-6-P is important for controlling glycogen accumulation in *Saccharomyces cerevisiae*. J Biol Chem 279:13764–13768

Pedreno Y, Gimeno-Alcaniz JV, Matallana E, Arguelles JC (2002) Response to oxidative stress caused by H(2)O(2) in *Saccharomyces cerevisiae* mutants deficient in trehalase genes. Arch Microbiol 177:494–499

Pedruzzi I, Burckert N, Egger P, De Virgilio C (2000) Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. EMBO J 19:2569–2579

Pedruzzi I, Dubouloz F, Cameroni E, Wanke V, Roosen J, Winderickx J, De Virgilio C (2003) TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. Mol Cell 12:1607–1613

Peng ZY, Trumbly RJ, Reimann EM (1990) Purification and characterization of glycogen synthase from a glycogen- deficient strain of *Saccharomyces cerevisiae*. J Biol Chem 265:13871–13877

Plourde-Owobi L, Durner S, Parrou JL, Wieczorke R, Goma G, Francois J (1999) *AGT1*, encoding an alpha-glucoside transporter involved in uptake and intracellular accumulation of trehalose in *Saccharomyces cerevisiae*. J Bacteriol 181:3830–3832

Posas F, Clotet J, Muns MT, Corominas J, Casamayor A, Arino J (1993) The gene PPG encodes a novel yeast protein phosphatase involved in glycogen accumulation. J Biol Chem 268:1349–1354

Ratnakumar S, Tunnacliffe A (2006) Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast. FEMS Yeast Res 6:902–913

Reinders A, Burckert N, Hohmann S, Thevelein JM, Boller T, Wiemken A, De Virgilio C (1997) Structural analysis of the subunits of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae* and their function during heat shock. Mol Microbiol 24:687–695

Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. Cell 111:17–28

Santhanam A, Hartley A, Duvel K, Broach JR, Garrett S (2004) PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. Eukaryot Cell 3:1261–1271

Schade B, Jansen G, Whiteway M, Entian KD, Thomas DY (2004) Cold adaptation in budding yeast. Mol Biol Cell 15:5492–5502

Sillje HHW, Paalman JWG, ter Schure EG, Olsthoorn SQB, Verkleij AJ, Boonstra J, Verrips CT (1999) Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*. J Bacteriol 181:396–400

Silva-Udawatta MN, Cannon JF (2001) Roles of trehalose phosphate synthase in yeast glycogen metabolism and sporulation. Mol Microbiol 40:1345–1356

Singer MA, Lindquist S (1998) Multiple effects of trehalose on protein folding in vitro and in vivo. Mol Cell 1:639–648

Skroch Stuart J, Frederick DL, Varner CM, Tatchell K (1994) The mutant type 1 protein phosphatase encoded by glc7-1 from *Saccharomyces cerevisiae* fails to interact productively with the GAC1- encoded regulatory subunit. Mol Cell Biol 14:896–905

Slattery MG, Liko D, Heideman W (2008) Protein kinase A, TOR, and glucose transport control the response to nutrient repletion in *Saccharomyces cerevisiae*. Eukaryot Cell 7:358–367

Smith TL, Rutter J (2007) Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. Mol Cell 26:491–499

Sunnarborg SW, Miller SP, Unnikrishnan I, LaPorte DC (2001) Expression of the yeast glycogen phosphorylase gene is regulated by stress-response elements and by the HOG MAP kinase pathway. Yeast 18:1505–1514

Teste MA, Enjalbert B, Parrou JL, Francois JM (2000) The Saccharomyces cerevisiae YPR184w gene encodes the glycogen debranching enzyme. FEMS Microbiol Lett 193:105–110

Teste MA, Duquenne M, Francois JM, Parrou JL (2009) Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. BMC Mol Biol 10:99

Thon VJ, Vigneron LC, Marianne PT, Montreuil J, Decq A, Rachez C, Ball SG, Cannon JF (1992) Coordinate regulation of glycogen metabolism in the yeast *Saccharomyces cerevisiae*. Induction of glycogen branching enzyme. J Biol Chem 267:15224–15228

Timblin BK, Bergman LW (1997) Elevated expression of stress response genes resulting from deletion of the *PHO85* gene. Mol Microbiol 26:981–990

Timblin BK, Tatchell K, Bergman LW (1996) Deletion of the gene encoding the cyclin-dependent protein kinase Pho85 alters glycogen metabolism in *Saccharomyces cerevisiae*. Genetics 143:57–66

Torija MJ, Novo M, Lemassu A, Wilson W, Roach PJ, Francois J, Parrou JL (2005) Glycogen synthesis in the absence of glycogenin in the yeast *Saccharomyces cerevisiae*. FEBS Lett 579:3999–4004

Tu BP, Kudlicki A, Rowicka M, McKnight SL (2005) Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. Science 310:1152–1158

Ugalde JE, Parodi AJ, Ugalde RA (2003) De novo synthesis of bacterial glycogen: agrobacterium tumefaciens glycogen synthase is involved in glucan initiation and elongation. Proc Natl Acad Sci USA 100:10659–10663

Van Aelst L, Hohmann S, Zimmermann FK, Jans AW, Thevelein JM (1991) A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisiae* mutant on fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling. EMBO J 10:2095–2104

van Voorst F, Houghton-Larsen J, Jonson L, Kielland-Brandt MC, Brandt A (2006) Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress. Yeast 23:351–359

Vandercammen A, Francois J, Hers HG (1989) Characterization of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *Saccharomyces cerevisiae*. Eur J Biochem 182:613–620

Versele M, Thevelein JM, Van Dijck P (2004) The high general stress resistance of the *Saccharomyces cerevisiae* fil1 adenylate cyclase mutant (Cyr1Lys1682) is only partially dependent on trehalose, Hsp104 and overexpression of Msn2/4-regulated genes. Yeast 21:75–86

Voit EO (2003) Biochemical and genomic regulation of the trehalose cycle in yeast: review of observations and canonical model analysis. J Theor Biol 223:55–78

Walther T, Novo M, Rossger K, Letisse F, Loret MO, Portais JC, Francois JM (2010) Control of ATP homeostasis during the respiro-fermentative transition in yeast. Mol Syst Biol 6:344

Wang Z, Wilson WA, Fujino MA, Roach PJ (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. Mol Cell Biol 21:5742–5752

Ward MP, Gimeno CJ, Fink GR, Garrett S (1995) SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. Mol Cell Biol 15:6854–6863

Wera S, De Schrijver E, Geyskens I, Nwaka S, Thevelein JM (1999) Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*. Biochem J 343:621–626

Wilson WA, Mahrenholz AM, Roach PJ (1999) Substrate targeting of the yeast cyclin-dependent kinase pho85p by the cyclin pcl10p. Mol Cell Biol 19:7020–7030

Wilson WA, Wang Z, Roach PJ (2002a) Analysis of respiratory mutants reveals new aspects of the control of glycogen accumulation by the cyclin-dependent protein kinase Pho85p. FEBS Lett 515:104–108

Wilson WA, Wang Z, Roach PJ (2002b) Systematic identification of the genes affecting glycogen storage in the yeast *Saccharomyces cerevisiae:* implication of the vacuole as a determinant of glycogen level. Mol Cell Proteomics 1:232–242

Wilson WA, Hughes WE, Tomamichel W, Roach PJ (2004) Increased glycogen storage in yeast results in less branched glycogen. Biochem Biophys Res Commun 320:416–423

Wilson WA, Wang Z, Roach PJ (2005) Regulation of yeast glycogen phosphorylase by the cyclin-dependent protein kinase Pho85p. Biochem Biophys Res Commun 329:161–167

Winderickx J, de WJ, Crauwels M, Hino A, Hohmann S, Van DP, Thevelein JM (1996) Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control. Mol Gen Genet 252:470–482

Wu X, Hart H, Cheng C, Roach PJ, Tatchell K (2001) Characterization of Gac1p, a regulatory subunit of protein phosphatase type I involved in glycogen accumulation in *Saccharomyces cerevisiae*. Mol Genet Genomics 265:622–635

Xu Z, Tsurugi K (2006) A potential mechanism of energy-metabolism oscillation in an aerobic chemostat culture of the yeast *Saccharomyces cerevisiae*. FEBS J 273:1696–1709

Xu Z, Tsurugi K (2007) Destabilization of energy-metabolism oscillation in the absence of trehalose synthesis in the chemostat culture of yeast. Arch Biochem Biophys 464:350–358

Yorimitsu T, Zaman S, Broach JR, Klionsky DJ (2007) Protein kinase A and Sch9 cooperatively regulate induction of autophagy in *Saccharomyces cerevisiae*. Mol Biol Cell 18:4180–4189

Zahringer H, Thevelein JM, Nwaka S (2000) Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol Microbiol 35:397–406

Zaman S, Lippman SI, Zhao X, Broach JR (2008) How *Saccharomyces* responds to nutrients. Annu Rev Genet 42:27–81

Zurita-Martinez SA, Cardenas ME (2005) Tor and cyclic AMP-protein kinase A: two parallel pathways regulating expression of genes required for cell growth. Eukaryot Cell 4:63–71

Molecular Mechanisms of Programmed Cell Death Induced by Acetic Acid in *Saccharomyces cerevisiae*

Sergio Giannattasio, Nicoletta Guaragnella, and Ersilia Marra

Contents

1 Introduction 58
2 Weak Acid Stress and Yeast Adaptation 58
2.1 Weak Acid Stress 59
2.2 Yeast Adaptation 60
3 Acetic Acid-Induced Programmed Cell Death 62
3.1 Mechanisms 62
3.2 Generation of Reactive Oxygen Species 64
3.3 The Role of Metacaspase-Encoding *YCA1* Gene 65
3.4 The Role of Mitochondria 66
4 Conclusions and Perspectives 69
References 70

Abstract Microorganisms face constant stressful conditions, such as weak acid stress, both in natural habitats and during their use for biotechnological applications. Microbes respond to stress by activating either cell adaptation or death pathways. Yeast *Saccharomyces cerevisiae* has been a valuable model to study the mechanisms of cell response to stressful environmental changes. This chapter summarizes current knowledge on molecular mechanisms of general weak acid stress response and programmed cell death in response to acetic acid as unraveled in *S. cerevisiae*. Future perspectives aimed at clarifying the complex intracellular signaling networks, integrating cell adaptation and death pathways in response to acetic acid stress are envisaged. Elucidation of finely regulated integration mechanisms of such pathways represents a challenge for understanding aspects of eukaryotic cell homeostasis as well as for improving the performance of a given yeast strain in industrial processes and applications.

S. Giannattasio (✉) • N. Guaragnella • E. Marra
CNR, Istituto di Biomembrane e Bioenergetica, Via Amendola 165/A, 70126 Bari, Italy
e-mail: s.giannattasio@ibbe.cnr.it

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_3,

1 Introduction

Microorganisms face constant stressful conditions, such as nutrient starvation, changes in temperature, osmolarity, and acidity of their surroundings both in natural habitats and in environments associated with their exploitation for biotechnological applications. Survival microbes react with alternatives in their genome expression and metabolism that lead to a physiological adaptation, allowing competitions under the newly evolved environmental conditions. Otherwise, the stressful conditions cause cell demise.

Weak acids, such as acetic, propionic, benzoic, and sorbic (*2,4*-hexadienoic) acid are widely used as food preservatives due to their well-known growth inhibitory effect on microorganisms including yeast. Numerous organic acid inhibitors have been identified for lignocellulosic biomass conversion to biofuels (Klinke et al. 2004; Liu and Blaschek 2010). Physiological effects of weak acids on microorganisms are depending on the composition and nature of monocarboxylate acid compound. Acids with more lipophilic moiety such as benzoate and sorbate usually cause delay of microbial cell growth and cytostasis. On the other hand, less lipophilic acetic acid under certain conditions compromises cell viability leading cells to death (Ludovico et al. 2001; Pinto et al. 1989).

Yeast *Saccharomyces cerevisiae* is one of the most thoroughly studied unicellular eukaryotes at the cellular, molecular, and genetic level due to their well-known experimental tractability. It is a valuable model to study the molecular mechanisms of cell response to stressful environmental changes (Gasch and Werner-Washburne 2002). In the past decade, evidence has also been gathered showing that *S. cerevisiae* is able to undergo a programmed cell death (PCD) process triggered by different internal and external stimuli. Such findings provide new tools and a model for cell death research at the molecular level (Carmona-Gutierrez et al. 2010a).

Considerable advances in weak acid response and adaptation mechanisms in *S. cerevisiae* have been achieved, and comprehensive reviews are available (Mollapour et al. 2008; Piper et al. 2001). This chapter summarizes the knowledge on the molecular mechanisms of general weak acid stress response and recent advances in the understanding of the mechanisms of PCD in response to acetic acid as unraveled in *S. cerevisiae*. Future perspectives aimed at clarifying the complex intracellular signaling networks, integrating adaptive and lethal responses to weak acid stress are presented.

2 Weak Acid Stress and Yeast Adaptation

Weak acid stress is a constant and major challenge to microbial life. It affects gene expression and metabolism for cell survival. This section provides an overview of weak acid stress response machinery activated in *S. cerevisiae* cells, leading to cell adaptation.

2.1 Weak Acid Stress

Weak acids display increased antimicrobial action at low pH in the undissociated state (Lambert and Stratford 1999). The uncharged molecules enter cells by simple diffusion mechanism through plasma membrane, encounter a more neutral pH in the cytoplasm, and dissociate into acid anions and protons. The protons lead to cytoplasmic acidification both in benzoate and acetate thereby inhibiting important metabolic processes (Arneborg et al. 2000; Krebs et al. 1983). Weak acid induces activation of the proton-translocating ATPase Pma1p in the plasma membrane of yeast, which pumps out the protons generated by weak acid dissociation in the cytosol in an ATP-dependent manner (see Fig. 1), thereby maintaining the electrochemical potential across plasma membrane regulating ion and pH balance and providing energy for nutrient uptake (Carmelo et al. 1996; Carmelo et al. 1997; Holyoak et al. 1996; Martinez-Munoz and Kane 2008).

Thus, intracellular acidification does not seem to be the exclusive cause of weak acid toxicity which seems to largely depend on the monocarboxylate anion. In fact, despite their identical pKa, higher concentrations of acetic acid such as 80–150 mM are needed to completely inhibit growth of *S. cerevisiae* than that of more lipophilic sorbic acid (1–3 mM) (Piper et al. 2001; Stratford and Anslow 1996, 1998). The differences in weak acid toxicity appear to mirror major differences existing in the transport of the weak acid and metabolism in yeast cells. Sorbate and benzoate cannot be metabolized by *S. cerevisiae* and have been shown to act as membrane-damaging substances (Stratford and Anslow 1998) and to cause severe oxidative stress under aerobic conditions (Piper et al. 2001; Piper 1999).

Benzoate inhibits glycolysis mainly at the phosphofructokinase reaction step (Krebs et al. 1983; Pearce et al. 2001). Strains of *Zygosaccharomyces bailii* are more resistant to weak acid stress; consistently, they are able to grow on benzoate, sorbate, and phenylalanine due to a benzoate-4-hydroxylase activity which is absent in *S. cerevisiae* (Mollapour and Piper 2001a; Mollapour and Piper 2001b). In a different way from benzoate, acetic acid can be used as the sole carbon and energy source by *S. cerevisiae* and is not toxic under such conditions. Thus, *S. cerevisiae* cells are normally able to grow on acetic acid medium at neutral pH. Under this condition, the weak acid is found in a dissociated form, and acetate is transported across the plasma membrane through a low-affinity electroneutral proton symport system that could transport propionate and formate but not lactate and pyruvate (Casal et al. 1996). Acetate uptaken by cells is used to form acetyl CoA by one of either peroxisomal or cytosolic acetyl-CoA synthetases. Acetyl-CoA is then consumed in the glyoxylate shunt or oxidized in mitochondria through the tricarboxylic acid cycle (Vilela-Moura et al. 2008 and refs. therein). However, typical *S. cerevisiae* cells grown on glucose cannot metabolize acetic acid due to the activation of glucose repression pathways responsible of down-regulation of respiration and gluconeogenesis (Rolland et al. 2002). Thus, yeast is sensitive to acetic acid stress. Acetate transport, as its metabolism, is also under glucose repression in *S. cerevisiae* but not in *Z. bailii* that is known for its high resistance to weak acids in glucose-containing media (Casal et al. 1996; Sousa et al. 1998).

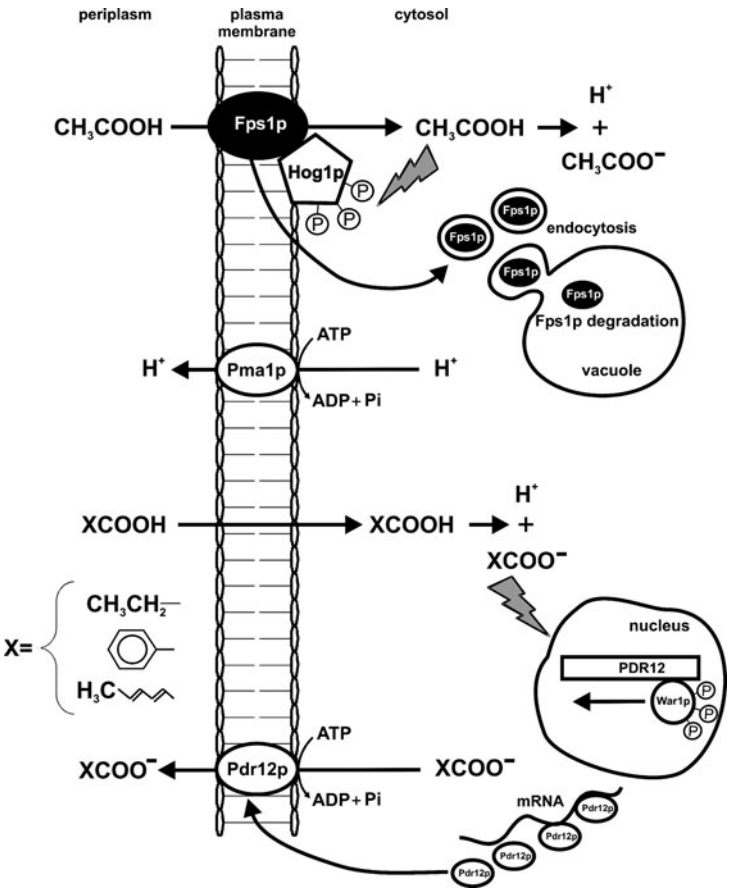

Fig. 1 Molecular mechanisms of weak acid adaptation in glucose-grown yeast involved in acetic acid, propionic acid, benzoic, and sorbic acid. Undissociated acetic acid enters cells through the plasma membrane aquaglyceroporin Fps1p and dissociates into acetate and protons in the cytosol. The Hog1p phosphorylation is induced by the acetic acid stress causing ubiquitination, endocytosis, and final degradation of Fps1p in the vacuole. Propionic, benzoic, and sorbic acids freely diffuse through the plasma membrane and dissociate into acid anions and protons in the cytosol. Intracellular acid anion pool activates *PDR12* transcription through phosphorylated War1p nuclear factor. This causes Pdr12p accumulation in the plasma membrane, which mediates extrusion of the acid anion leading to weak acid stress adaptation. Intracellular acidification due to the proton accumulation is counteracted by the activity of H^+-ATPase Pma1p, which pumps out protons with energy requirement

2.2 Yeast Adaptation

When challenged by weak acid stress, *S. cerevisiae* cells are able to adjust transcriptional programs enabling a rapid tuning of protein expression patterns (Schuller et al. 2004). Under certain conditions, yeast cells can activate an adaptive response and resume to grow after a lag phase. Mechanisms of yeast adaptation to

most common monocarboxylate preservatives are mainly involved in plasma membrane transporters and proton-translocating ATPase together with cell organelles involved in weak acid stress response (Fig. 1).

Plasma membrane transporter Pdr12p, a member of ATP-binding cassette (ABC) transporter family was strongly induced by sorbate, benzoate, and certain other moderately lipophilic carboxylate compounds, but not organic alcohols or high levels of acetate on glucose-containing medium at pH 4.5 (Hatzixanthis et al. 2003; Piper et al. 1998). The accumulation of Pdr12p in the plasma membrane increases sorbate resistance mediating cellular extrusion of weak acid anion (Piper et al. 1998). The adaptation involves induction of *PDR12* gene transcription and requires a nuclear transcription factor War1p which binds the cis-acting weak acid response element (WARE) located at the promoter region of *PDR12* (Kren et al. 2003). War1p forms homodimers that constitutively binds DNA and is phosphorylated under the sorbate stress (Fig. 1). A genetic screen for the isolation of yeast mutant cells that failed to induce *PDR12* allowed identification of *WAR1* mutations and confirmed War1p as the major regulator of *PDR12* for the stress response (Gregori et al. 2007).

Using a screening of a gene deletion library in combination with transcriptome profiling analysis, more than 100 genes were found to be induced by sorbic acid stress (Schuller et al. 2004). Many of these genes are regulated by transcription factors Msn2p/Msn4p involved in the general stress response pathway and/or War1p. Additional sets of genes activated by the sorbate stress were also identified. Another transcription factor Haa1p involved in transcription activation in response to acetaldehyde has been shown to be required for a rapid adaptation by yeast to weak acids such as acetic and propionic acids (Fernandes et al. 2005). It is likely that PDR12 is required and inducible for weak acid resistance. At least, a fraction of a given genomic response is necessary to cope with adverse conditions caused by weak acids (Schuller et al. 2004).

Unlike the sorbate stress, in which a gain of function is involved in the acid resistance through the induction of *PDR12*, adaptation to acetic acid involves a loss of function (Mollapour et al. 2008 and refs. therein) involving another monocarboxylate transporter (Fig. 1). At pH 4.5, acetic acid has been demonstrated to enter glucose-repressed *S. cerevisiae* cells in its undissociated form primarily by facilitated diffusion through the Fps1p aquaglyceroporin channel (Mollapour and Piper 2007). Acetic acid challenge at low pH causes activation of two mitogen-activated protein (MAP) kinases, Hog1p, involved in the high-osmolarity glycerol (HOG) signaling pathway (Hohmann 2009), and Slt2p, involved in cell wall integrity pathway (Fuchs and Mylonakis 2009). Only the loss of Hog1p, not Slt2p, increases the yeast sensitivity to acetate. The Hog1p-mediated acetic acid adaptation has been shown involving direct MAP kinase Hog1p-dependent phosphorylation of Fps1p that result in its ubiquitination, endocytosis, and final degradation in the vacuole (Mollapour and Piper 2007) (Fig. 1). Hog1p exists in physical association with the *N*-terminal cytosolic domain of Fps1p in unstressed cells, and the presence of Fps1p is essential for Hog1p activation, exerting opposing effects

on Hog1p, and Slt2p MAP kinases in *S. cerevisiae* exposed to acetic acid stress (Mollapour et al. 2009).

In fact, such a weak acid stress response is different from the adaptation to the hyperosmotic stress. At pH 6.8 on glucose medium cultures, acetic acid is almost entirely dissociated to the acetate anion, and inhibition of *S. cerevisiae* cell growth is observed at very high concentrations of acetate for example, 500 mM. This condition induces a typical HOG response to sodium acetate salt stress (Hohmann 2009; Mollapour and Piper 2006). In this case, the expression of *GPD1*, encoding glycerol-3-phosphate dehydrogenase catalyzing the first step of glycerol biosyntheis from dihydroxyacetonephosphate, is induced with the increased intracellular glycerol level to counteract hyperosmotic stress. At pH 4.5, acetic acid is substantially undissociated; a much lower acetate level (100 mM) is needed to cause comparable growth inhibition, with *GPD1* transcript displaying only a slight, transient induction and declining of intracellular glycerol (Mollapour and Piper 2006). Therefore, in a weak acid-specific manner, the Hog1p-directed destabilization of Fps1p eliminates the route for acetic acid entry to the cell, generating a resistance to varied levels of acetic acid that would otherwise prove toxic (Mollapour et al. 2008).

3 Acetic Acid-Induced Programmed Cell Death

Under certain conditions, yeast *S. cerevisiae* undergoes a programmed cell death process in response to lethal concentrations of acetic acid. Recent achievements in the characterization of cell components and mechanisms involved in yeast acetic acid-induced programmed cell death are discussed below.

3.1 Mechanisms

The term *programmed cell death* (PCD) describes a highly heterogeneous process regulated by distinct but sometimes overlapping pathways including apoptosis, autophagic cell death, and necrosis according to their morphology (Kroemer et al. 2009). Apoptosis is activated in multicellular organisms, like mammals, with a diverse physiological role, as it is in normal development, cell differentiation, immune response, stress response, and the demise of damaged cells. Morphological hallmarks of apoptotic cells include cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and membrane "blebbing," culminating in the formation of apoptotic bodies which are eventually removed by phagocytosis (engulfment). In addition, apoptosis is inhibited by the protein synthesis inhibitor cycloheximide. A variety of pro- and anti-apoptotic factors is involved in the onset and execution of mammalian apoptosis, including plasma membrane receptors and mitochondrial proteins. Indeed, mitochondria play a pivotal role in apoptosis as the

receivers, integrators, and transmitters of death signals (Goldenthal and Marin-Garcia 2004).

Since the discovery of a yeast mutant exhibiting apoptosis hallmarks (Madeo et al. 1997), compelling evidence has been gathered showing that the unicellular eukaryote *S. cerevisiae* can undergo a PCD process. Thus, due to the high degree of conservation of genes and proteins between *S. cerevisiae* and higher eukaryotes, yeast has been established as a model system to investigate how PCD occurs, in particular to identify regulatory pathways responsible for physiological and pathological processes in eukaryotes. Yeast PCD, which is triggered by a variety of endogenous and exogenous stimuli including gene mutations, aging, and heterologous expression of human pro-apoptotic proteins, shares most of the biochemical and morphological hallmarks of mammalian apoptosis, including cycloheximide inhibition, nuclear condensation, and DNA fragmentation (see Carmona-Gutierrez et al. 2010a).

As described above, it is known that under certain conditions acetic acid can cause cell demise in yeast (Pinto et al. 1989). In an attempt to characterize the mode of cell death occurring in glucose-repressed yeast cells exposed to acetic acid at pH 3.0, it was found that *S. cerevisiae* commits to a PCD process in response to 20–80 mM acetic acid (AA-PCD) (Ludovico et al. 2002; Ludovico et al. 2001; Ribeiro et al. 2006). Interestingly, *Z. bailii*, known to be more weak acid resistant than *S. cerevisiae*, was also shown to undergo the AA-PCD but only in response to higher concentrations of acetic acid at 320–800 mM (Ludovico et al. 2003). It needs to be pointed out that when acetic acid was used at concentration higher than 80 mM, *S. cerevisiae* cell death was not inhibited by cycloheximide and showed ultrastructural alterations typical of necrosis (Ludovico et al. 2001).

Apparent, physiologically relevant mechanisms of the PCD are present in yeast which function as important regulators for yeast cell populations (Gourlay et al. 2006; Severin et al. 2008; Vachova and Palkova 2005). In nature, *S. cerevisiae* can be found in acidic environments such as rotten fruit and other decomposed plant materials. When exploited for biotechnology applications, yeasts and other competitor microbes are able to produce monocarboxylic acids, including acetic acid, as end products of metabolism with a consequent acidification of their surroundings. Thus, the capability of *S. cerevisiae* to cope with acetic acid stress should be assumed as a physiological behavior. On the other hand, *S. cerevisiae* cell suicide has been suggested to be physiologically relevant to increase the fitness of the whole cell population (Longo et al. 2005; Severin et al. 2008), as an altruistic role of yeast cell death. In this context, the gradual acidification of the culture medium due to accumulation of acetic acid in the aged culture as a result of glycolysis might contribute to a quorum-sensing mechanism (Knorre et al. 2005). Consistently, acetic acid has been shown to accumulate in aged yeast cultures and to be the primary cause of chronological aging in a population of nondividing yeast cells (Burhans and Weinberger 2009; Burtner et al. 2009).

At present, many yeast genes and proteins, orthologues of mammalian apoptosis regulators, have been identified, and their roles investigated in yeast cell death pathways (Carmona-Gutierrez et al. 2010a; Frohlich et al. 2007; Greenwood and

Ludovico 2009). However, certain processes occurred in mammalian apoptosis were not observed as the same manner in yeast PCD induced by different stimuli such as acetic acid. These processes include reactive oxygen species (ROS) accumulation, activation of proteolytic systems such as metacaspase, and the release of pro-apoptotic mitochondrial proteins, e.g., cytochrome *c* (cyt *c*) to the cytoplasm (Carmona-Gutierrez et al. 2010a; Eisenberg et al. 2007; Madeo et al. 2009; Pereira et al. 2008).

3.2 Generation of Reactive Oxygen Species

The generation of ROS is a common feature of the PCD in a variety of organisms, where the ROS can either activate pathways to save the cell from demise or impair the cellular redox balance or trigger the PCD. Numerous apoptotic stimuli, including the addition of hydrogen peroxide or acetic acid, glutathione depletion, hyperosmotic stress of high glucose concentration, and pheromone and amiodarone, cause increased ROS production in yeast cells (Ludovico et al. 2002; Madeo et al. 1999; Pozniakovsky et al. 2005; Silva et al. 2005). The key role of ROS in the commitment of yeast cells to PCD is now largely recognized, and many questions concerning the relationships between yeast apoptosis and ROS generation can be fully addressed. The main questions are as follows: What ROS are involved in cell death process, and where are they generated? What are the target/s of the ROS, and how are they targeted? Are ROS directly triggering AA-PCD or as secondary products of the apoptotic cascade (Perrone et al. 2008)? A study on the role of oxidative stress in yeast cells *en route* to AA-PCD has been carried out with the aim to gain insight into these issues. It has been shown that acetic acid leads to early intracellular H_2O_2 accumulation with the increased H_2O_2 levels occurring at 15 min after death induction. Then the H_2O_2 levels decrease after 60 min when they are undetectable. On the other hand, accumulation of superoxide anion is observed only at a later time (90 min) (Guaragnella et al. 2007). The observed difference in the time course of H_2O_2 and superoxide anion is in favor of a different role for the two species during AA-PCD.

The level of intracellular ROS is under the control of the antioxidant system, including superoxide dismutase (SOD) and catalase, responsible for scavenging superoxide anion and hydrogen peroxide, respectively. Activities of the SOD and catalase have been assayed *en route* to AA-PCD. The SOD activity increases after AA addition, reaching a maximum at 15 min, and decreases afterward. On the other hand, the catalase activity is undetectable during the AA-PCD (Giannattasio et al. 2005). Whether the catalase undergoes enzyme inactivation and/or degradation in the AA-PCD cells remain to be elucidated. Although autophagic programmed cell death occurs as a result of selective catalase degradation in mouse cell lines (Yu et al. 2006), autophagy has shown not to be activated in the AA-PCD (Pereira et al. 2010).

Catalase has a protective function under several stress conditions in *S. cerevisiae* (Schuller et al. 1994). Accordingly, in cells overexpressing cytosolic catalase, encoded by CTT1 gene, the AA-PCD was prevented (Guaragnella et al. 2008). In these cells, a lower level of H_2O_2 was detected compared to the control cells. In cells overexpressing cytosolic SOD, encoded by SOD1 gene, the AA-PCD was exacerbated and H_2O_2 levels were higher than the control cells. Together, these data suggested a major role for H_2O_2 in modulating yeast cell response to acetic acid.

Confirmation of the protective role of the catalase in *S. cerevisiae* AA-PCD is that yeast cells develop an adaptive response to the AA-PCD when exposed to extracellular acidification at pH 3.0 (Giannattasio et al. 2005). Under these conditions, high levels of both SOD and catalase activities with low levels of both superoxide anion and H_2O_2 were found (Guaragnella et al. 2007). In general, these data indicate a role of H_2O_2 acting as a second messenger to start the apoptotic cascade triggered by acetic acid. The relationships among the ROS and other biochemical events of the AA-PCD, including cyt *c* release and caspase activation (see below) have been investigated. Either cyt *c* release or caspase activation resulted to be inhibited by the antioxidant *N*-acetyl-*L*-cysteine (Guaragnella et al. 2010b), which further supports the role of H_2O_2 causing the AA-PCD.

At what cellular levels and how acetic acid leads to the intracellular superoxide and H_2O_2 generation remains to be investigated. Certainly mitochondria are the major source of ROS in the AA-PCD (Eisenberg et al. 2007; Ludovico et al. 2002). Moreover, it has been proposed that the occurrence of intracellular acidification, following acetic acid treatment, causes superoxide protonation to $HO_2^{\bullet}$, which is one of the most aggressive ROS. In this death cascade, the protein Ysp2p, localized into mitochondria, has been shown to act downstream of ROS and play a major role in mediating mitochondrial thread-to-grain transition *en route* to the PCD (Sokolov et al. 2006). Such a process proved to be a necessary step in various types of apoptosis (Frank et al. 2001), including yeast PCD induced by acetic acid or H_2O_2 (Fannjiang et al. 2004). Impairment of cytochrome *c* oxidase has been shown *en route* to the AA-PCD (Giannattasio et al. 2008; Pereira et al. 2007). It might also cause the increased ROS production in AA-PCD cells (Richter et al. 1995).

3.3 *The Role of Metacaspase-Encoding* YCA1 *Gene*

Proteolytic systems are the major executors of degradation of cell components in mammalian apoptosis. Caspases are cysteine proteases playing a crucial, but sometimes facultative, role in the initiation and execution of most cell death pathways in higher eukaryotes (Atlante et al. 2003; Leist and Jaattela 2001). Two caspase-related protein families have been identified: paracaspases, found both in animals and other organisms lacking caspases, and metacaspases, found in plants, fungi, and protozoa (Uren et al. 2000). *S. cerevisiae* contains only one metacaspase encoded by *YCA1* gene, with its protein product regulating the PCD process (Madeo et al. 2002). However, regarding to the mode of induction for the PCD, either

YCA1-dependent or *YCA1*-independent pathways have been recognized in yeast (Madeo et al. 2009).

Metacaspase of *S. cerevisiae* shows cleavage specificity different from that of caspases since it can hydrolyze proteins after arginine or lysine residues, but not after aspartate (Watanabe and Lam 2005). Although most targets of the yeast metacaspase are unknown, the phylogenetically conserved Tudor staphyloccocal nuclease has recently shown to be the first metacaspase substrate to be identified in plants; interestingly, it was shown to be cleaved also by caspase-3 in mammals and to have a role in programmed cell death in both organisms (Sundstrom et al. 2009). Nonetheless, the clear role and functions of metacaspase in yeast PCD remain unknown (Carmona-Gutierrez et al. 2010b). As far as the AA-PCD is concerned, *YCA1*-lacking cells have been shown to undergo PCD as observed in wild-type cells, but with a lower death rate. A caspase-like activity has been shown to be specifically activated *en route* to AA-PCD in a late phase (200 min) and to be dependent on *YCA1*. However, caspase-like activity inhibition does not increase cell viability upon the AA-PCD induction, showing that *YCA1* participates in the AA-PCD independently from the caspase-like activity (Guaragnella et al. 2010a; Guaragnella et al. 2006). On the other hand, *YCA1*-independent caspase activities have also been measured *en route* to yeast AA-PCD (Guaragnella et al. 2010a; Hauptmann and Lehle 2008).

Proteasomal degradation system has also been implicated in the AA-PCD, a proteasome transient activation being necessary for the AA-PCD (Valenti et al. 2008). Although acetic acid induces an early burst of H_2O_2 in *YCA1* and/or cyt *c* knockout yeast cells, it activates a ROS-independent AA-PCD pathway (Guaragnella et al. 2010a; Guaragnella et al. 2010b). Deletion of the caspase-like gene has shown to lead to high intracellular ROS level or a large accumulation of oxidized proteins upon PCD induction with formic acid or H_2O_2, respectively (Du et al. 2008; Khan et al. 2005). Cells of Δyca1 mutant were shown to accumulate deleterious mutations with time (Severin et al. 2008). However, the basic mechanism responsible for these changes remains to be established.

3.4 The Role of Mitochondria

In addition to the crucial functions for energy production and metabolic pathways, mitochondria play a key role in integrating cell death stimuli and executing the apoptotic program. They are the major source of the ROS (see above) and release crucial *pro-death factors*, including AIF, ENDO G, and cyt *c*. The involvement of mitochondria in yeast PCD has been largely recognized (Eisenberg et al. 2007; Pereira et al. 2008). As far as the AA-PCD is concerned, the first evidence of cyt *c* release has been reported (Ludovico et al. 2002). In a series of experiments carried out to ascertain how cyt *c* was released *en route* to the AA-PCD, it was shown that it starts at 60 min of the AA-PCD and completes at 150 min. Degradation of the released cyt *c* occurs later possibly due to unidentified proteases

(Giannattasio et al. 2008). Since mitochondria are proven to be coupled when cyt *c* has already been released, the question arises as to the role of the released cyt *c* in the AA-PCD. In agreement with Atlante et al. (2003), it has been shown that the released cyt *c* functions both as a ROS scavenger and a respiratory substrate (Giannattasio et al. 2008). This is consistent with the antioxidant functions proposed for cyt *c* in the apoptotic cascade (Skulachev 1998) together with its role in supplying energy for the AA-PCD execution.

Although the involvement of the ADP/ATP carrier for mitochondrial outer membrane permeabilization and cyt *c* release has been reported (Pereira et al. 2007), mechanisms underlying cyt *c* release in the AA-PCD remain to be elucidated. Interestingly, changes in the mitochondrial morphology similar to mammalian thread-to-grain transition (Skulachev et al. 2004) have been observed during the AA-PCD, with fragmented mitochondria subsequently removed in a late phase of the death process (Fannjiang et al. 2004). This suggests the involvement of phylogenetically conserved mitochondrial fission/division proteins in the release of mitochondrial proteins to the cytosol during yeast PCD (Cheng et al. 2008). Vacuolar protease Pep4p has shown to be released to the cytosol and playing a role, together with the ADP/ATP carrier, in mitochondrial degradation in yeast cells undergoing AA-PCD (Pereira et al. 2010). A progressive impairment in mitochondrial functions was also observed *en route* to the AA-PCD: collapse of the membrane potential and gradual uncoupling, with a decrease in cytochrome *c* oxidase activity and in the amounts of cytochrome *c* oxidase subunit II and of cytochromes $a + a_3$. (Giannattasio et al. 2008; Ludovico et al. 2002).

Due to its genetic tractability, yeast serves as a powerful tool to study both mechanism and the regulation of the PCD. In this regard, the AA-PCD has been investigated in *YCA1* and/or *CYC1* and *CYC7*-lacking cells. *CYC1* and *CYC7* encode for the two isoforms of cyt *c* in yeast. As stated above, *YCA1*-lacking cells undergo the AA-PCD with typical apoptotic hallmarks, but with a death rate slower than that of the wild type. Since no cyt *c* release occurs in these cells, it indicates the involvement of *YCA1* in the cyt *c* release during the AA-PCD. Further investigation is needed to confirm the *YCA1* functions in this process. The evidence of cell death of the cyt *c*-lacking cells via PCD following acetic acid treatment clearly shows that cyt *c* release is dispensable for the AA-PCD (Guaragnella et al. 2010a).

All these findings are in favor of the existence of at least two death pathways induced by yeast cell treatment with acetic acid: the *YCA1*-dependent and *YCA1*-independent AA-PCD. Of course, mitochondria still play a major role in the *YCA1*-independent PCD in which no cyt *c* release takes place. Aif1p, an orthologue of apoptosis-inducing factor AIF involved in caspase-independent mammalian apoptosis in *S. cerevisiae* (Joza et al. 2009) is required for the AA-PCD to occur; in particular, *en route* to death Aif1p moves from mitochondria to the nucleus. Its function in PCD has been shown to be partially dependent on *YCA1* (Wissing et al. 2004). Thus, mitochondria play a different role in the two described AA-PCD pathways (Fig. 2). In mammalian PCD, cyt *c* is a component of the apoptosome which in turn promotes caspase activation (Riedl and Salvesen 2007). No evidence

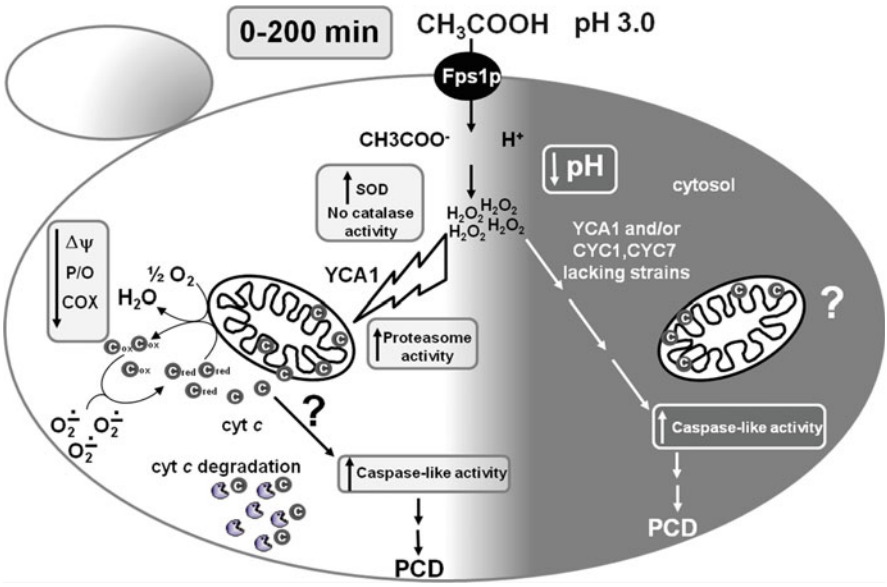

Fig. 2 Yeast acetic acid-programmed cell death in glucose-grown cells. Acetic acid is assumed to enter yeast cells by facilitated diffusion through the plasma membrane aquaglyceroporin Fps1p. In the cytosol, acetic acid dissociates into acetate and protons causing intracellular acidification. Alternative PCD pathways are induced by acetic acid in wild type (*white background*) and *YCA1* and/or *CYC1* and *CYC7* deletion mutants (*gray background*). Hydrogen peroxide (H_2O_2) accumulates earlier with the increase of superoxide dismutase (*SOD*) activity. Cyt *c* (*c*) is released from mitochondria to the cytosol and acts as an electron donor (c_{red}) to mitochondrial respiratory chain and as superoxide anion ($O_2^{\cdot-}$) scavenger (c_{ox}); cyt *c* is degraded by unidentified proteases in a late phase. Mitochondrial functions are progressively declined as judged by decrease in mitochondrial membrane potential ($\Delta\psi$) P/O ratio and cytochrome *c* oxidase (*COX*) activity. *YCA1* is required for cyt *c* release. A caspase-like activity is increased in a late phase with a complete loss of cell viability at 200 min. In the *YCA1*-independent AA-PCD pathway, cyt *c* is not released into the cytosol, but the caspase-like activity is increased (see text for details)

of an apoptosome-like structure in yeast has been reported thus far. Nevertheless, a complex mechanism in regulation of the caspase-like activity *en route* to the AA-PCD is expected to exist. Accordingly, different effects on caspase-like activities have been observed in AA-PCD depending on the variety of yeast mutations. Specifically, an early and extra activation of caspase activity has been observed in cyt *c*-lacking cells (Guaragnella et al. 2010a) which is a subject of continued investigation.

Compounds, genes, proteins, and their interrelations in *S. cerevisiae en route* to the AA-PCD are schematically shown in Fig. 2. Alternative PCD pathways induced by acetic acid in wild type and *YCA1* and/or *CYC1* and *CYC7* lacking strains are also indicated. Yeast cells lost viability 200 min after challenges with a lethal concentration of acetic acid on a glucose medium. Acetic acid enters cells by facilitated transport through plasma membrane aquaglyceroporin Fps1p causing intracellular acidification. A specific and early intracellular high level of H_2O_2 is

detected at 15 min and then decreased and was undetectable at 60 min. In wild-type cells, the proteasome activation starts at 60 min after the AA-PCD induction, with a maximum at 90 min, and decreases at 150 min. The release of cyt *c* starts at 60 min of the AA-PCD when 60% cells remain alive and completes at 150 min. The released cyt *c* functions as an electron donor and a ROS scavenger. At a late stage, there is a gradual decrease in mitochondrial coupling with a decrease in $\Delta\psi$ and an impairment of cytochrome *c* oxidase (COX); the released cyt *c* is degraded at 200 min. Caspase-like activity progressively increases up to a maximum at 200 min. For the mutant yeast cells, cyt *c* is not released *en route* to AA-PCD, but a late caspase-like activity increase is observed.

4 Conclusions and Perspectives

The occurrence of an orchestrated course of events triggered by acetic acid and leading either to cell adaptation to weak acid stress or to cell demise is illustrated in *S. cerevisiae*. Yet, the relations between yeast PCD regulators and components of the intracellular signaling cascade activated by acetic acid stress remain unknown. Recent findings on yeast adaptation response under the acidic stress to protect yeast cells from the AA-PCD (Giannattasio et al. 2005) are of special interest. The Hog1p-dependent degradation of Fps1p has been hypothesized as a mechanism of the protection from the AA-PCD (Mollapour et al. 2008). However, acetic acid is present in the Hog1p-mediated weak acid stress adaptation mechanism as described above (Fig. 1), whereas it was absent in the low pH medium used in the previous observations (Giannattasio et al. 2005). A possible activation of the general stress response pathway inducing *CTT1* gene expression, as reported in yeast cells grown in low pH media by HCl (Schuller et al. 1994), is more consistent with the observed increase of the intracellular catalase activity. Investigation on the role of certain signaling pathways in *S. cerevisiae* AA-PCD has been initiated. Target of rapamycin (TOR) kinase signaling pathway that regulates cell growth in response to nutrient availability, has been shown to be involved in the AA-PCD (Almeida et al. 2009). As mentioned above, acetic acid has been identified as an extracellular mediator of cell death during chronological aging in yeast (Burhans and Weinberger 2009; Burtner et al. 2009). This process involves the RAS-cAMP-PKA and the SCH9 signaling pathways, which are known to control yeast cell adaptation to nutrient availability as well as chronological lifespan in yeast (Longo 2003; Roosen et al. 2005). The SCH9 is a major component of TOR pathway (Urban et al. 2007). Consistently, intracellular acidification, induced by weak acids on a low pH medium, stimulates the RAS-cAMP signaling pathway, negatively regulating cell viability (Colombo et al. 1998; Lastauskiene and Citavicius 2008). Thus, understanding the complex intracellular regulatory network integrating cell adaptation and death pathways in response to weak acid stress is a challenge for future investigations, which will shed light on many aspects of eukaryotic cell homeostasis. *S. cerevisiae* will continue to serve as an ideal eukaryotic model

organism to unravel mechanisms involved in degenerative processes, answering fundamental questions such as those regarding the different responses to apoptotic stimuli of cells in a population, depending on variation in cellular environment as well as cell adaptation and cell death in response to stress. Applications of yeast in more innovative utilizations of biorefineries involve more challenges of numerous environmental stresses (Kvitek et al. 2008; Scheckhuber et al. 2009). Ultimately, improving the efficiency of stress response in a given yeast strain determines "its robustness, and to a large extent, whether it is able to perform to necessary commercial standards in industrial processes" (Attfield 1997).

Acknowledgements We thank Professor Salvatore Passarella for critical reading of the manuscript. This work was financially supported by a grant from Fondazione Cassa di Risparmio di Puglia and Program FIRB-MERIT [1-RBNE08HWLZ_012] and [1-RBNE08YFN3_005].

References

Almeida B, Ohlmeier S, Almeida AJ, Madeo F, Leao C, Rodrigues F, Ludovico P (2009) Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway. Proteomics 9:720–732

Arneborg N, Jespersen L, Jakobsen M (2000) Individual cells of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* exhibit different short-term intracellular pH responses to acetic acid. Arch Microbiol 174:125–128

Atlante A, de Bari L, Bobba A, Marra E, Calissano P, Passarella S (2003) Cytochrome c, released from cerebellar granule cells undergoing apoptosis or excytotoxic death, can generate protonmotive force and drive ATP synthesis in isolated mitochondria. J Neurochem 86:591–604

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357

Burhans WC, Weinberger M (2009) Acetic acid effects on aging in budding yeast: are they relevant to aging in higher eukaryotes? Cell Cycle 8:2300–2302

Burtner CR, Murakami CJ, Kennedy BK, Kaeberlein M (2009) A molecular mechanism of chronological aging in yeast. Cell Cycle 8:1256–1270

Carmelo V, Bogaerts P, Sa-Correia I (1996) Activity of plasma membrane H+−ATPase and expression of PMA1 and PMA2 genes in *Saccharomyces cerevisiae* cells grown at optimal and low pH. Arch Microbiol 166:315–320

Carmelo V, Santos H, Sa-Correia I (1997) Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. Biochim Biophys Acta 1325:63–70

Carmona-Gutierrez D, Eisenberg T, Buttner S, Meisinger C, Kroemer G, Madeo F (2010a) Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ. doi:10.1038/cdd.2009.219

Carmona-Gutierrez D, Frohlich KU, Kroemer G, Madeo F (2010b) Metacaspases are caspases. Doubt no more. Cell Death Differ 17:377–378

Casal M, Cardoso H, Leao C (1996) Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. Microbiology 142(Pt 6):1385–1390

Cheng WC, Leach KM, Hardwick JM (2008) Mitochondrial death pathways in yeast and mammalian cells. Biochim Biophys Acta 1783:1272–1279

Colombo S, Ma P, Cauwenberg L, Winderickx J, Crauwels M, Teunissen A, Nauwelaers D, de Winde JH, Gorwa MF, Colavizza D, Thevelein JM (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. EMBO J 17:3326–3341

Du L, Su Y, Sun D, Zhu W, Wang J, Zhuang X, Zhou S, Lu Y (2008) Formic acid induces Yca1p-independent apoptosis-like cell death in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 8:531–539

Eisenberg T, Buttner S, Kroemer G, Madeo F (2007) The mitochondrial pathway in yeast apoptosis. Apoptosis 12:1011–1023

Fannjiang Y, Cheng WC, Lee SJ, Qi B, Pevsner J, McCaffery JM, Hill RB, Basanez G, Hardwick JM (2004) Mitochondrial fission proteins regulate programmed cell death in yeast. Genes Dev 18:2785–2797

Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sa-Correia I (2005) *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun 337:95–103

Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1:515–525

Frohlich KU, Fussi H, Ruckenstuhl C (2007) Yeast apoptosis-From genes to pathways. Semin Cancer Biol 17:112–121

Fuchs BB, Mylonakis E (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot Cell 8:1616–1625

Gasch AP, Werner-Washburne M (2002) The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics 2:181–192

Giannattasio S, Guaragnella N, Corte-Real M, Passarella S, Marra E (2005) Acid stress adaptation protects *Saccharomyces cerevisiae* from acetic acid-induced programmed cell death. Gene 354:93–98

Giannattasio S, Atlante A, Antonacci L, Guaragnella N, Lattanzio P, Passarella S, Marra E (2008) Cytochrome c is released from coupled mitochondria of yeast en route to acetic acid-induced programmed cell death and can work as an electron donor and a ROS scavenger. FEBS Lett 582:1519–1525

Goldenthal MJ, Marin-Garcia J (2004) Mitochondrial signaling pathways: a receiver/integrator organelle. Mol Cell Biochem 262:1–16

Gourlay CW, Du W, Ayscough KR (2006) Apoptosis in yeast - mechanisms and benefits to a unicellular organism. Mol Microbiol 62:1515–1521

Greenwood MT, Ludovico P (2009) Expressing and functional analysis of mammalian apoptotic regulators in yeast. Cell Death Differ 17:737–745

Gregori C, Bauer B, Schwartz C, Kren A, Schuller C, Kuchler K (2007) A genetic screen identifies mutations in the yeast WAR1 gene, linking transcription factor phosphorylation to weak-acid stress adaptation. FEBS J 274:3094–3107

Guaragnella N, Pereira C, Sousa MJ, Antonacci L, Passarella S, Corte-Real M, Marra E, Giannattasio S (2006) YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. FEBS Lett 580:6880–6884

Guaragnella N, Antonacci L, Passarella S, Marra E, Giannattasio S (2007) Hydrogen peroxide and superoxide anion production during acetic acid-induced yeast programmed cell death. Folia Microbiol 7:237–240

Guaragnella N, Antonacci L, Giannattasio S, Marra E, Passarella S (2008) Catalase T and Cu, Zn-superoxide dismutase in the acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. FEBS Lett 582:210–214

Guaragnella N, Bobba A, Passarella S, Marra E, Giannattasio S (2010a) Yeast acetic acid-induced programmed cell death can occur without cytochrome c release which requires metacaspase YCA1. FEBS Lett 584:224–228

Guaragnella N, Passarella S, Marra E, Giannattasio S (2010b) Knock-out of metacaspase and/or cytochrome c results in the activation of a ROS-independent acetic acid-induced programmed cell death pathway in yeast. FEBS Lett 584:3655–3660

Hatzixanthis K, Mollapour M, Seymour I, Bauer BE, Krapf G, Schuller C, Kuchler K, Piper PW (2003) Moderately lipophilic carboxylate compounds are the selective inducers of the *Saccharomyces cerevisiae* Pdr12p ATP-binding cassette transporter. Yeast 20:575–585

Hauptmann P, Lehle L (2008) Kex1 protease is involved in yeast cell death induced by defective N-glycosylation, acetic acid, and chronological aging. J Biol Chem 283:19151–19163

Hohmann S (2009) Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. FEBS Lett 583:4025–4029

Holyoak CD, Stratford M, McMullin Z, Cole MB, Crimmins K, Brown AJ, Coote PJ (1996) Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. Appl Environ Microbiol 62:3158–3164

Joza N, Pospisilik JA, Hangen E, Hanada T, Modjtahedi N, Penninger JM, Kroemer G (2009) AIF: not just an apoptosis-inducing factor. Ann NY Acad Sci 1171:2–11

Khan MA, Chock PB, Stadtman ER (2005) Knockout of caspase-like gene, YCA1, abrogates apoptosis and elevates oxidized proteins in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 102:17326–17331

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Knorre DA, Smirnova EA, Severin FF (2005) Natural conditions inducing programmed cell death in the yeast *Saccharomyces cerevisiae*. Biochem (Mosc) 70:264–266

Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F (1983) Studies on the mechanism of the antifungal action of benzoate. Biochem J 214:657–663

Kren A, Mamnun YM, Bauer BE, Schuller C, Wolfger H, Hatzixanthis K, Mollapour M, Gregori C, Piper P, Kuchler K (2003) War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol Cell Biol 23:1775–1785

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16:3–11

Kvitek DJ, Will JL, Gasch AP (2008) Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. PLoS Genet 4:e1000223

Lambert RJ, Stratford M (1999) Weak-acid preservatives: modelling microbial inhibition and response. J Appl Microbiol 86:157–164

Lastauskiene E, Citavicius D (2008) Influence of RAS genes on yeast *Saccharomyces cerevisiae* cell viability in acidic environment. Biologija 54:150–155

Leist M, Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2:589–598

Liu ZL, Blaschek HP (2010) Biomass conversion inhibitors and in situ detoxification. In: Vertes A, Qureshi N, Yukawa H, Blaschek H (eds) Biomass to biofuels: strategies for global industries. Wiley, UK, pp 233–259

Longo VD (2003) The Ras and Sch9 pathways regulate stress resistance and longevity. Exp Gerontol 38:807–811

Longo VD, Mitteldorf J, Skulachev VP (2005) Programmed and altruistic ageing. Nat Rev Genet 6:866–872

Ludovico P, Sousa MJ, Silva MT, Leao C, Corte-Real M (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147:2409–2415

Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A, Corte-Real M (2002) Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Mol Biol Cell 13:2598–2606

Ludovico P, Sansonetty F, Silva MT, Corte-Real M (2003) Acetic acid induces a programmed cell death process in the food spoilage yeast *Zygosaccharomyces bailii*. FEMS Yeast Res 3:91–96

Madeo F, Frohlich E, Frohlich KU (1997) A yeast mutant showing diagnostic markers of early and late apoptosis. J Cell Biol 139:729–734

Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145:757–767

Madeo F, Herker E, Maldener C, Wissing S, Lachelt S, Herlan M, Fehr M, Lauber K, Sigrist SJ, Wesselborg S, Frohlich KU (2002) A caspase-related protease regulates apoptosis in yeast. Mol Cell 9:911–917

Madeo F, Carmona-Gutierrez D, Ring J, Buttner S, Eisenberg T, Kroemer G (2009) Caspase-dependent and caspase-independent cell death pathways in yeast. Biochem Biophys Res Commun 382:227–231

Martinez-Munoz GA, Kane P (2008) Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J Biol Chem 283:20309–20319

Mollapour M, Piper P (2001a) Targeted gene deletion in *Zygosaccharomyces bailii*. Yeast 18:173–186

Mollapour M, Piper PW (2001b) The ZbYME2 gene from the food spoilage yeast *Zygosaccharomyces bailii* confers not only YME2 functions in *Saccharomyces cerevisiae*, but also the capacity for catabolism of sorbate and benzoate, two major weak organic acid preservatives. Mol Microbiol 42:919–930

Mollapour M, Piper PW (2006) Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. FEMS Yeast Res 6:1274–1280

Mollapour M, Piper PW (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol Cell Biol 27:6446–6456

Mollapour M, Shepherd A, Piper PW (2008) Novel stress responses facilitate *Saccharomyces cerevisiae* growth in the presence of the monocarboxylate preservatives. Yeast 25:169–177

Mollapour M, Shepherd A, Piper PW (2009) Presence of the Fps1p aquaglyceroporin channel is essential for Hog1p activation, but suppresses Slt2(Mpk1)p activation, with acetic acid stress of yeast. Microbiology 155:3304–3311

Pearce AK, Booth IR, Brown AJ (2001) Genetic manipulation of 6-phosphofructo-1-kinase and fructose 2,6-bisphosphate levels affects the extent to which benzoic acid inhibits the growth of *Saccharomyces cerevisiae*. Microbiology 147:403–410

Pereira C, Camougrand N, Manon S, Sousa MJ, Corte-Real M (2007) ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis. Mol Microbiol 66:571–582

Pereira C, Silva RD, Saraiva L, Johansson B, Sousa MJ, Corte-Real M (2008) Mitochondria-dependent apoptosis in yeast. Biochim Biophys Acta 1783:1286–1302

Pereira C, Chaves S, Alves S, Salin B, Camougrand N, Manon S, Joao Sousa M, Corte-Real M (2010) Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4 and the ADP/ATP carrier. Mol Microbiol. doi:10.1111/j.1365-2958.2010.07122.x

Perrone GG, Tan SX, Dawes IW (2008) Reactive oxygen species and yeast apoptosis. Biochim Biophys Acta 1783:1354–1368

Pinto I, Cardoso H, Leao C, van Uden N (1989) High enthalpy and low enthalpy death in *Saccharomyces cerevisiae* induced by acetic acid. Biotechnol Bioeng 33:1350–1352

Piper PW (1999) Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radic Biol Med 27:1219–1227

Piper P, Mahe Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Muhlbauer M, Coote P, Kuchler K (1998) The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 17:4257–4265

Piper P, Calderon CO, Hatzixanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147:2635–2642

Pozniakovsky AI, Knorre DA, Markova OV, Hyman AA, Skulachev VP, Severin FF (2005) Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. J Cell Biol 168:257–269

Ribeiro GF, Corte-Real M, Johansson B (2006) Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. Mol Biol Cell 17:4584–4591

Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P, Yaffee M (1995) Oxidants in mitochondria: from physiology to diseases. Biochim Biophys Acta 1271:67–74

Riedl SJ, Salvesen GS (2007) The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 8:405–413

Rolland F, Winderickx J, Thevelein JM (2002) Glucose-sensing and -signalling mechanisms in yeast. FEMS Yeast Res 2:183–201

Roosen J, Engelen K, Marchal K, Mathys J, Griffioen G, Cameroni E, Thevelein JM, De Virgilio C, De Moor B, Winderickx J (2005) PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. Mol Microbiol 55:862–880

Scheckhuber CQ, Mitterbauer R, Osiewacz HD (2009) Molecular basis of and interference into degenerative processes in fungi: potential relevance for improving biotechnological performance of microorganisms. Appl Microbiol Biotechnol 85:27–35

Schuller C, Brewster JL, Alexander MR, Gustin MC, Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. EMBO J 13:4382–4389

Schuller C, Mamnun YM, Mollapour M, Krapf G, Schuster M, Bauer BE, Piper PW, Kuchler K (2004) Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. Mol Biol Cell 15:706–720

Severin FF, Meer MV, Smirnova EA, Knorre DA, Skulachev VP (2008) Natural causes of programmed death of yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1783:1350–1353

Silva RD, Sotoca R, Johansson B, Ludovico P, Sansonetty F, Silva MT, Peinado JM, Corte-Real M (2005) Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. Mol Microbiol 58:824–834

Skulachev VP (1998) Cytochrome c in the apoptotic and antioxidant cascades. FEBS Lett 423:275–280

Skulachev VP, Bakeeva LE, Chernyak BV, Domnina LV, Minin AA, Pletjushkina OY, Saprunova VB, Skulachev IV, Tsyplenkova VG, Vasiliev JM, Yaguzhinsky LS, Zorov DB (2004) Thread-grain transition of mitochondrial reticulum as a step of mitoptosis and apoptosis. Mol Cell Biochem 256–257:341–358

Sokolov S, Knorre D, Smirnova E, Markova O, Pozniakovsky A, Skulachev V, Severin F (2006) Ysp2 mediates death of yeast induced by amiodarone or intracellular acidification. Biochim Biophys Acta 1757:1366–1370

Sousa MJ, Rodrigues F, Corte-Real M, Leao C (1998) Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. Microbiology 144(Pt 3):665–670

Stratford M, Anslow PA (1996) Comparison of the inhibitory action on *Saccharomyces cerevisiae* of weak-acid preservatives, uncouplers, and medium-chain fatty acids. FEMS Microbiol Lett 142:53–58

Stratford M, Anslow PA (1998) Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. Lett Appl Microbiol 27:203–206

Sundstrom JF, Vaculova A, Smertenko AP, Savenkov EI, Golovko A, Minina E, Tiwari BS, Rodriguez-Nieto S, Zamyatnin AA Jr, Valineva T, Saarikettu J, Frilander MJ, Suarez MF,

Zavialov A, Stahl U, Hussey PJ, Silvennoinen O, Sundberg E, Zhivotovsky B, Bozhkov PV (2009) Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. Nat Cell Biol 11:1347–1354

Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN, Loewith R (2007) Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. Mol Cell 26:663–674

Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell 6:961–967

Vachova L, Palkova Z (2005) Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. J Cell Biol 169:711–717

Valenti D, Vacca RA, Guaragnella N, Passarella S, Marra E, Giannattasio S (2008) A transient proteasome activation is needed for acetic acid-induced programmed cell death to occur in *Saccharomyces cerevisiae*. FEMS Yeast Res 8:400–404

Vilela-Moura A, Schuller D, Mendes-Faia A, Corte-Real M (2008) Reduction of volatile acidity of wines by selected yeast strains. Appl Microbiol Biotechnol 80:881–890

Watanabe N, Lam E (2005) Two Arabidopsis metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast. J Biol Chem 280:14691–14699

Wissing S, Ludovico P, Herker E, Buttner S, Engelhardt SM, Decker T, Link A, Proksch A, Rodrigues F, Corte-Real M, Frohlich KU, Manns J, Cande C, Sigrist SJ, Kroemer G, Madeo F (2004) An AIF orthologue regulates apoptosis in yeast. J Cell Biol 166:969–974

Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M (2006) Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci USA 103:4952–4957

Molecular Mechanisms of Ethanol Tolerance in *Saccharomyces cerevisiae*

Menggen Ma and Z. Lewis Liu

Contents

1 Introduction 78
2 Expression of Structure and Organelle Related Genes 79
3 Membrane and Cell Wall 79
  3.1 Vacuole 87
  3.2 Mitochondrion 87
  3.3 Peroxisome 88
4 Amino Acid Encoding Genes 88
  4.1 Tryptophan 88
  4.2 Proline 94
5 Heat Shock Proteins 95
  5.1 Chaperones 95
  5.2 Other Functions 97
6 Pathway Analysis 99
  6.1 Trehalose and Glycogen Metabolisms 99
  6.2 Glycolysis and Fermentation 101
  6.3 Pentose Phosphate Pathway 101
7 Cofactor Homeostasis and Ion Transport 102
  7.1 Cofactor Redox Balance 102
  7.2 Ion Transport 102
8 Regulatory Networks 103
  8.1 Signal Transduction Pathways 103
  8.2 Transcription Factors and Stress Response Element 105
9 Conclusions and Perspectives 108
References 108

M. Ma (✉) • Z.L. Liu
Bioenergy Research, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA
e-mail: mgen23@hotmail.com

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_4,

Abstract The yeast *Saccharomyces cerevisiae* is a superb ethanol producer, yet sensitive to ethanol at higher concentrations, especially under high gravity or very high gravity fermentation conditions. Although significant efforts have been made to study ethanol stress response in past decades, molecular mechanisms of ethanol tolerance are not well known. With developments of genome sequencing and genomic technologies, our understanding of yeast biology has been revolutionarily advanced. Additional evidence of ethanol tolerance has been discovered involving numerous genes with variety of functions, multiple loci, and complex interactions, as well as signal transduction pathways and regulatory networks. Genetic manipulation of one or a few genes is unable to achieve desirable phenotype for multiple stress tolerance. Transcription dynamics and profiling studies of key gene sets such as heat shock proteins provided new insight into tolerance mechanisms. A transient gene expression response or a stress response to ethanol does not necessarily lead to ethanol-tolerant phenotype in yeast. Reprogrammed pathways and interactions of cofactor regeneration and redox balance revealed by time-course studies suggest constitutive gene expression response is important for ethanol tolerance. Fine-tuned expression of key transcription factor genes, which regulate numerous genes associated with ethanol stress, may achieve desirable phenotype and avoid side effect to cell growth at the same time.

1 Introduction

The yeast *Saccharomyces cerevisiae* has been widely used for alcohol related brewing and fermentation for thousands of years (Legras et al. 2007). In recent years, with increasing price of fossil oil and its accelerating depletion, bioethanol production for transportation energy has received widespread attention due to its renewable and sustainable productivity, as well as reduction of air pollution, and greenhouse gas, CO_2, for global warming (Outlaw et al. 2005; Liu et al. 2008; Sánchez and Cardona 2008; Vertes et al. 2009). Low-cost and high-titer of ethanol production are vital challenges in bio-based economy development. To achieve cost-efficient production of bioethanol, high gravity or very high gravity fermentation technologies to produce high concentrations of ethanol is promising due to its reduction of capital, energy, distillation, and labor costs. *S. cerevisiae* is a desirable ethanol producer among numerous fermentative microorganisms (Lin and Tanaka 2006; Liu et al. 2008). However, it is sensitive to high concentrations of ethanol. Ethanol diffuses freely across biological membranes in yeast cells allowing equalization of ethanol concentrations between intracellular and extracellular of cells. As a result, the increased ethanol concentration inhibits cell growth, affects cell viability, and reduces ethanol fermentation rate and final yield (Casey and Ingledew 1986; D'Amore and Stewart 1987; D'Amore et al. 1990; Bai et al. 2004; Pina et al. 2004; Ding et al. 2009). At high concentrations, ethanol has been shown to perturb protein conformation causing protein denaturation and dysfunction (Millar et al. 1982; Pascual et al. 1988); affect uptake of glucose, maltose, ammonium, and

amino acids; and cause leakage of nucleotides, amino acids, and potassium (Piper 1995).

Developing high ethanol-tolerant strain is desired for bioethanol production from biomass. Ethanol tolerance varies in yeast strains. Some strains are able to accumulate ethanol up to 20% in final fermentation (Hara et al. 1976a, b; Ogawa et al. 2000). Molecular mechanisms of ethanol tolerance have been studied for decades. Several hundred genes were identified to be associated with ethanol tolerance, which involves a broad range of functional categories, including membrane and cell wall organization, heat shock proteins, amino acid metabolism, nucleotide metabolism, transport, cell cycle and growth, lipid metabolism, fatty acid, and ergosterol metabolism (Gasch et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Kubota et al. 2004; Fujita et al. 2006; van Voorst et al. 2006; Auesukaree et al. 2009; Dinh et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009; Ma and Liu 2010a). Every single gene showing induced expression by ethanol possesses multiple functions (Tables 1 and 2) that further complicate interpretation of gene interactions and relationships. This chapter mainly focuses on our current understanding of molecular mechanisms to ethanol tolerance based on comprehensive gene expression and regulatory network analyses.

2 Expression of Structure and Organelle Related Genes

Under ethanol stress conditions, remodeling of cell structures and organelles occurs to maintain cell functions. Expression dynamics of many genes involved in cell wall, membrane, vacuole, mitochondrion, and peroxisome reflect such functions to ethanol tolerance.

3 Membrane and Cell Wall

Cell membranes, especially plasma membrane, are considered as main target sites of ethanol (D'Amore and Stewart 1987). Many genes involving membrane composition were identified to be associated with ethanol tolerance. Monounsaturated fatty acids, including palmitoleic acid and oleic acid in the *S. cerevisiae*, are key plasma membrane components to compensate deficits caused by ethanol stress through increasing the fluidity of the plasma membrane. Both palmitoleic acid and oleic acid were formed by the same catabolic membrane desaturase encoded by *OLE1* through oxygen- and NADH-dependent desaturation of palmitic acid and stearic acid, respectively (Stukey et al. 1989, 1990). The amount of these two unsaturated fatty acids (UFAs) in cellular lipids was higher in ethanol-tolerant strains (Sajbidor et al. 1995; You et al. 2003). Oleic acid is considered as the main determinant of ethanol tolerance by supplementation with synthetic monounsaturated fatty acids to medium and expression of insect desaturase TniNPVE in

Table 1 Gene Ontology (GO) categories and terms for significantly induced genes by ethanol in S*accharomyces cerevisiae*

GO ID	GO term	Gene(s) annotated to the term
Cellular component		
GO:0005737	Cytoplasm	*SSA1, CDC19, PRX1, SSA3, ATG8, NTH2, ETR1, YRO2, HSP26, TPS1, YBR139W, ADH5, RTC2, TOS1, SSE2, SDS24, YBR287W, GRX1, GLK1, YCL042W, PDI1, CIT2, PGK1, GPM2, GPD1, YDL124W, STF1, SFA1, COS7, NTH1, TPS2, SED1, HSP42, SDH4, YDR248C, HSP78, CCC2, HXT7, HXT6, GRX2, EMI2, EUG1, GLC3, UBC8, SPF1, CYC7, YEL047C, PRB1, YAT2, PIC2, GIP2, HOR2, RGI1, SER3, SSA4, COX15, HSP12, GSY1, HXK1, PNC1, PKP2, PYC1, STF2, CTT1, RTS3, TDH3, PDX1, PCT1, SOL4, ENO1, GND2, COS6, AIM17, SOD2, ARG4, GRE3, ENO2, CTR2, OYE2, PFK26, YJL016W, TDH1, MPM1, KHA1, OPI3, UGP1, LHS1, LAP4, MCR1, YKL151C, SSA2, HSP104, TPO1, UBI4, AHP1, PUT1, CPR6, GSY2, ATP14, DAK1, ATP18, TSL1, ERO1, ADH3, PGM2, ALD2, ICY1, HOR7, ADH2, PBI2, APJ1, YNL134C, CIT1, DDR2, ATP19, ADH1, MCH4, GRE2, GCY1, SRL1, RDL1, FAA1, ALD4, IRC15, SSE1, HSP82, ATH1, GPH1, GDB1*
GO:0005739	Mitochondrion	*PRX1, NTH2, ETR1, YRO2, RTC2, CIT2, PGK1, STF1, SFA1, COS7, TPS2, SED1, SDH4, HSP78, HXT7, HXT6, GRX2, SPF1, CYC7, YEL047C, PIC2, COX15, GSY1, HXK1, PKP2, STF2, TDH3, PDX1, ENO1, AIM17, SOD2, ENO2, OYE2, TDH1, MPM1, KHA1, OPI3, MCR1, SSA2, PUT1, ATP14, ATP18, ADH3, APJ1, CIT1, ATP19, RDL1, FAA1, ALD4, GDB1*
GO:0016020	Membrane	*SSA1, ATG8, AGP1, HSP30, PMP1, STF1, SDH4, CCC2, HXT7, HXT6, PDR15, SPF1, COX15, DDI1, HSP12, STF2, ENO2, CTR2, KHA1, MCR1, PTR2, SSA2, TPO1, YPS3, ATP14, ATP18, ICY1, HOR7, MEP2, ATP19, MCH4, RDL1, FAA1, SSU1, DIP5*
GO:0005634	Nucleus	*SSA1, APN2, HSP26, ADH5, GRX1, RPN4, YDL124W, HOR2, RGI1, SSA4, GRX4, HSP12, PNC1, NQM1, RTS3, PCT1, SOL4, COS8, PCL5, GRE3, OYE2, PHD1, HSP104, HHT2, APJ1, YNL134C, GRE2, GCY1, GSP2*
GO:0005624	Membrane fraction	*SSA1, CDC19, GLK1, PGK1, HSP30, YDL124W, HXT7, HXT6, YEL047C, HSP12, TDH3, ENO1, GND2, ENO2, TDH1, MPM1, UGP1, PTR2, SSA2, AHP1, HOR7, ADH1, FAA1*
GO:0005773	Vacuole	*SSA1, ATG8, YBR139W, TOS1, PRB1, ENO1, COS6, ENO2, CTR2, LAP4, SSA2, TPO1, ICY1, PBI2, DDR2, MCH4, SRL1, ATH1*

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s) annotated to the term
GO:0005886	Plasma membrane	*AGP1,HSP30,PMP1,HXT7,HXT6,DDI1,HSP12, ENO2,PTR2,TPO1,YPS3,HOR7,MEP2,SSU1, DIP5*
GO:0005618	Cell wall	*SSA1, TIP1, TOS1, SED1, SPI1, TDH3, FLO5, TDH1, HSP150, SSA2, YPS1, HOR7, SRL1, ATH1*
GO:0005740	Mitochondrial envelope	*STF1, SDH4, CYC7, COX15, STF2, MCR1, ATP14, ATP18, ATP19, RDL1, FAA1*
GO:0005783	Endoplasmic reticulum	*YBR287W, PDI1, EUG1, SPF1, OPI3, LHS1, ERO1, HOR7, RDL1*
GO:0012505	Endomembrane system	*CCC2, SPF1, PCT1, COS8*
GO:0005777	Peroxisome	*CIT2, GPD1, PNC1*
GO:0005794	Golgi apparatus	*CCC2, PCT1, KHA1*
GO:0005933	Cellular bud	*YRO2, TPO1, SRL1*
GO:0005840	Ribosome	*SED1, YEL047C*
GO:0005856	Cytoskeleton	*HSP42, IRC15*
GO:0005576	Extracellular region	*HSP150, YGP1*
GO:0016023	Cytoplasmic membrane-bounded vesicle	*CCC2*
GO:0030427	Site of polarized growth	*SRL1*
GO:0005694	Chromosome	*HHT2*
GO:0005575	Cellular component unknown	*YCR013C, YDR133C, YFL066C, YGL117W, YGR146C, PAU13, YHL050C, YKL044W, GLG1, YMR018W, YOL157C, SIA1, OYE3, HSP32*
Other	Other	*ADH7, HSP31, UGA1*
Biological process		
GO:0006950	Response to stress	*SSA1, APN2, PRX1, SSA3, ATG8, HSP26, TPS1, GRX1, HSP30, RPN4, GPD1, YDL124W, NTH1, TPS2, HSP42, HSP78, GRX2, PRB1, HOR2, SSA4, GRX4, HSP12, STF2, CTT1, SOD2, GRE3, LHS1, MCR1, SSA2, HSP104, UBI4, AHP1, DAK1, TSL1, HOR7, DDR2, GRE2, GCY1, HSP82, ATH1*
GO:0044262	Cellular carbohydrate metabolic process	*CDC19, NTH2, TPS1, GLK1, CIT2, PGK1, NTH1, TPS2, YDR248C, GLC3, UBC8, SPF1, GIP2, HOR2, GSY1, HXK1, PYC1, TDH3, SOL4, ENO1, GND2, ENO2, PFK26, TDH1, UGP1, GLG1, HSP104, GSY2, DAK1, TSL1, PGM2, ATH1, GPH1, GDB1*
GO:0006810	Transport	*SSA1, SSA3, ATG8, SDS24, AGP1, GLK1, PMP1, HSP78, CCC2, HXT7, HXT6, PDR15, SPF1, PIC2, SSA4, DDI1, HXK1, CTR2, KHA1, LHS1, PTR2, SSA2, TPO1, ATP14, ATP18, MEP2, ATP19, MCH4, SIA1, FAA1, SSU1, HSP82, DIP5*

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s) annotated to the term
GO:0006091	Generation of precursor metabolites and energy	*CDC19, ETR1, ADH5, GLK1, PGK1, SDH4, GLC3, CYC7, GIP2, HOR2, GSY1, HXK1, TDH3, ENO1, ENO2, PFK26, TDH1, UGP1, GLG1, GSY2, ATP14, ATP18, ADH3, PGM2, ADH2, CIT1, ATP19, ADH1, GPH1, GDB1*
GO:0042221	Response to chemical stimulus	*PRX1, TPS1, TOS1, GRX1, RPN4, YDL124W, PDR15, GRX2, EMI2, SPI1, GRX4, HSP12, CTT1, SOD2, GRE3, LHS1, MCR1, HSP104, AHP1, GCY1*
GO:0006519	Cellular amino acid and derivative metabolic process	*ADH5, CIT2, SFA1, YAT2, SER3, UGA1, PCT1, ARG4, OPI3, PUT1, ADH3, ALD2, ADH2, CIT1, ADH1*
GO:0006457	Protein folding	*SSA1, SSA3, HSP26, SSE2, PDI1, HSP78, EUG1, SSA4, SSA2, HSP104, CPR6, ERO1, SSE1, HSP82*
GO:0051186	Cofactor metabolic process	*ADH5, GPD1, SDH4, COX15, PNC1, PYC1, PDX1, SOL4, GND2, ADH3, ADH2, CIT1, ADH1, ALD4*
GO:0006766	Vitamin metabolic process	*ADH5, GPD1, YAT2, PNC1, PYC1, SOL4, GND2, ADH3, ADH2, ADH1, ALD4*
GO:0019725	Cellular homeostasis	*PRX1, GRX1, GPD1, CCC2, GRX2, SPF1, GRX4, AHP1, PGM2*
GO:0006464	Protein modification process	*UBC8, SPF1, GIP2, PKP2, UGP1, UBI4, ERO1, FAA1*
GO:004255	Cellular lipid metabolic process	*ETR1, PCT1, OPI3, MCR1, GRE2, FAA1*
GO:0046483	Heterocycle metabolic process	*SFA1, COX15, PUT1, ATP14, ATP18, ATP19*
GO:0006350	Transcription	*RPN4, EMI2, PNC1, PCL5, PHD1*
GO:0016044	Membrane organization	*ATG8, SDS24, ENO1, ENO2, PBI2*
GO:0045333	Cellular respiration	*ETR1, SDH4, CYC7, CIT1*
GO:0007005	Mitochondrion organization	*SSA1, SED1, HSP78, HSP82*
GO:0007047	Cell wall organization	*TIP1, SED1, HSP150, YPS3*
GO:0006259	DNA metabolic process	*APN2, RPN4, IRC15, HSP82*
GO:0044257	Cellular protein catabolic process	*UBC8, PRB1, DDI1, LAP4*
GO:0007033	Vacuole organization	*ENO1, ENO2, PBI2*
GO:0051276	Chromosome organization	*HHT2, IRC15, HSP82*
GO:0030435	Sporulation resulting in formation of a cellular spore	*EMI2, PRB1, UBI4*
GO:0016070	RNA metabolic process	*EMI2,PNC1,PHD1*
GO:0016192	Vesicle-mediated transport	*ATG8, SDS24, DDI1*
GO:0007010	Cytoskeleton organization	*HSP42, IRC15*
GO:0007049	Cell cycle	*RPN4, IRC15*
GO:0007124	Pseudohyphal growth	*PHD1, MEP2*
GO:0006412	Translation	*SSA1, HSP78*
GO:0000910	Cytokinesis	*SDS24*

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s) annotated to the term
GO:0007059	Chromosome segregation	*IRC15*
GO:0007126	Meiosis	*IRC15*
GO:0016050	Vesicle organization	*ATG8*
GO:0006997	Nucleus organization	*GSP2*
GO:0070271	Protein complex biogenesis	*HSP82*
GO:0006725	Cellular aromatic compound metabolic process	*YDL124W*
GO:0008150	Biological process unknown	*YRO2, RTC2, YBR287W, YCL042W, YCR013C, GPM2, COS7, YDR133C, HSP31, RGI1, YFL066C, YGL117W, NQM1, YGR146C, RTS3, COS6, AIM17, PAU13, COS8, YHL050C, OYE2, YJL016W, MPM1, YKL044W, YKL151C, YMR018W, ICY1, APJ1, YNL134C, YOL157C, RDL1, OYE3, HSP32*
Other	Other	*YBR139W, ADH7, STF1, YEL047C, FLO5, YPS1, YGP1, SRL1*
Molecular function		
GO:0016491	Oxidoreductase activity	*PRX1, ETR1, ADH5, GRX1, PDI1, ADH7, GPD1, YDL124W, SFA1, SDH4, GRX2, EUG1, YEL047C, SER3, COX15, GRX4, CTT1, TDH3, GND2, SOD2, GRE3, OYE2, TDH1, MCR1, AHP1, PUT1, ERO1, ADH3, ALD2, ADH2, YNL134C, ADH1, GRE2, GCY1, ALD4, OYE3*
GO:0016787	Hydrolase activity	*SSA1, APN2, SSA3, NTH2, TIP1, YBR139W, NTH1, TPS2, HSP78, CCC2, PDR15, HSP31, SPF1, PRB1, HOR2, SSA4, PNC1, SOL4, YHL050C, LAP4, SSA2, HSP104, YPS1, YPS3, ATP14, TSL1, GSP2, HSP82, HSP32, ATH1, GDB1*
GO:0016740	Transferase activity	*CDC19, TPS1, GRX1, GLK1, CIT2, PGK1, YDR248C, GRX2, GLC3, YAT2, GSY1, HXK1, PKP2, UGA1, NQM1, PCT1, PFK26, OPI3, UGP1, GLG1, GSY2, DAK1, TSL1, CIT1, IRC15, GPH1, GDB1*
GO:0005515	Protein binding	*SSA1, SSA3, HSP26, HSP42, HSP78, HSP31, SSA4, DDI1, PDX1, LHS1, SSA2, HSP104, UBI4, CPR6, APJ1, IRC15, HSP82, HSP32*
GO:0005215	Transporter activity	*AGP1, CCC2, HXT7, HXT6, PDR15, SPF1, PIC2, CTR2, KHA1, PTR2, TPO1, ATP14, ATP18, MEP2, ATP19, MCH4, SSU1, DIP5*
GO:0030234	Enzyme regulator activity	*SSE2, PMP1, GIP2, PCL5, LHS1, TSL1, PBI2, SSE1*
GO:0008233	Peptidase activity	*YBR139W, HSP31, PRB1, LAP4, YPS1, YPS3, HSP32*
GO:0016853	Isomerase activity	*PDI1, GPM2, EUG1, CPR6, PGM2*
GO:0016829	Lyase activity	*APN2, ENO1, ARG4, ENO2*
GO:0005198	Structural molecule activity	*TIP1, SED1, HSP150, YPS3*
GO:0016874	Ligase activity	*UBC8, PYC1, FAA1*
GO:0003677	DNA binding	*RPN4, PHD1 ,HHT2*

(continued)

Table 1 (continued)

GO ID	GO term	Gene(s) annotated to the term
GO:0016779	Nucleotidyltransferase activity	*PCT1, UGP1*
GO:0030528	Transcription regulator activity	*RPN4, PHD1*
GO:0004672	Protein kinase activity	*PKP2*
GO:0004871	Signal transducer activity	*COS7*
GO:0004386	Helicase activity	*YHL050C*
GO:0003674	Molecular function unknown	*ATG8, YRO2, RTC2, TOS1, SDS24, YBR287W, YCL042W, YCR013C, HSP30, GPM2, STF1, YDR133C, EMI2, RGI1, SPI1, HSP12, YFL066C, YGL117W, STF2, YGR146C, RTS3, COS6, AIM17, PAU13, COS8, YJL016W, MPM1, YKL044W, YKL151C, YMR018W, ICY1, HOR7, YGP1, DDR2, YOL157C, SIA1, SRL1, RDL1*
Other	Other	*CYC7, FLO5*

Source: Data from Ogawa et al. (2000), Alexandre et al. (2001), Chandler et al. (2004), Marks et al. (2008), Dinh et al. (2009), and Ma and Liu (2010a). Function of gene products was classified using Gene Ontology (GO) Slim Mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl)

S. cerevisiae (You et al. 2003). *ELO1* and *OLE1* encode enzyme in the important steps for oleic acid synthesis. Expression of *ELO1* was enhanced by ethanol in ethanol-tolerant strain Y-50316 (Ma and Liu 2010a). Although transcription of *OLE1*, encoding enzyme for the last step of oleic acid biosynthesis, was repressed by ethanol, its transcription level in ethanol-tolerant strain was significantly higher than its parental control. Ergosterol is one of the major components in cellular membrane associated with plasma membrane fluidity. Higher ergosterol content in yeast was found to be associated with higher ethanol tolerance (del Castillo Agudo 1992). This was further confirmed by defective growth of deletion mutants under ethanol stress, including *ERG2*, *ERG3*, *ERG5*, *ERG6*, *ERG24*, and *ERG28*; all involved in ergosterol biosynthesis (Kubota et al. 2004; Fujita et al. 2006; Van voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009). In addition, *ETR1*, *GPD1*, *DAK1*, *PCT1*, *OPI3*, *MCR1*, *FAA1*, and *GRE2* involved in fatty acid, lipid, and isoprenoid metabolism were reported to be up-regulated under ethanol stress (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a). Except for de novo biosynthesis of fatty acids, *S. cerevisiae* is also able to import a variety of exogenous saturated and poly-unsaturated fatty acids from the growth medium and incorporate them into membrane lipids rapidly (Choi et al. 1996; Xiao et al. 2010).

Ethanol-tolerant mutants K11 and SR4-3, tolerant up to 20% ethanol, showed strong resistance to a cell wall lysis enzyme zymolyase, suggesting the cell wall's function in ethanol tolerance (Ogawa et al. 2000). Genome-wide studies uncovered that up-regulated genes involving cell wall structure under ethanol stress include *TIP1* for mannoprotein metabolism, *SED1* for glycoprotein metabolism, *SPI1* for weak acid resistance, and *HSP150* for cell wall organization (Ogawa et al. 2000;

Table 2 Functional categories and terms of significantly induced genes by ethanol challenge for *Saccharomyces cerevisiae* based on gene products classified according to Functional Catalogue described in Munich Information Center for Protein Sequences (MIPS) database

MIPS ID	Functionary category	*p*-value	Entries
01.05.02.04	Sugar, glucoside, polyol, and carboxylate anabolism	2.70E-07	**NTH2**, **TPS1**, **NTH1**, **NQM1**, **PFK26**, **UGP1**, **TSL1**, **PGM2**, **ATH1**
01.05.02.07	Sugar, glucoside, polyol, and carboxylate catabolism	2.26E-11	**CDC19**, **NTH2**, **TPS1**, **PGK1**, **NTH1**, **SDH4**, **NQM1**, **TDH3**, **ENO1**, **GRE3**, **ENO2**, **PFK26**, **TDH1**, **UGP1**, **PGM2**, CIT1, **ATH1**
01.05.03.01	Glycogen metabolism	1.94E-04	**GSY1**, **GLG1**, **GSY2**
2.01	Glycolysis and gluconeogenesis	1.96E-12	**CDC19**, GLK1, **PGK1**, **ADH7**, UBC8, HXK1, PYC1, **TDH3**, PDX1, **ENO1**, **ENO2**, **PFK26**, **TDH1**, **PGM2**, YNL134c, **GRE2**
2.07	Pentose phosphate pathway	3.77E-03	**NQM1**, SOL4, **GND2**, **PGM2**
2.11	Electron transport and membrane-associated energy conservation	2.30E-04	**STF1**, **SDH4**, **CYC7**, **STF2**, **MCR1**, **ATP14**, **ATP18**, **ATP19**
2.13	Respiration	3.69E-04	ETR1, **STF1**, **SDH4**, **CYC7**, **YEL047c**, COX15, **STF2**, **MCR1**, **ATP14**, **ATP18**, **ATP19**, **ALD4**
2.16	Fermentation	2.56E-04	**ADH5**, **ADH7**, ADH3, **ALD2**, ADH2, ADH1, **ALD4**
2.19	Metabolism of energy reserves (e.g., glycogen, trehalose)	4.79E-14	**NTH2**, **TPS1**, **NTH1**, **TPS2**, GLC3, GIP2, **GSY1**, **UGP1**, **GLG1**, **GSY2**, **TSL1**, **PGM2**, YOL157c, **HSP82**, **ATH1**, GPH1, GDB1
2.45	Energy conversion and regeneration	2.06E-05	**STF1**, **STF2**, **OYE2**, **ATP14**, **ATP18**, **ALD2**, **ATP19**, **OYE3**
14.01	Protein folding and stabilization	3.51E-10	**SSA1**, **SSA3**, **HSP26**, **SSE2**, PDI1, **HSP42**, **HSP78**, EUG1, **SSA4**, **LHS1**, SSA2, **HSP104**, **CRP6**, **ERO1**, **APJ1**, **SSE1**, **HSP82**
16.21	Complex cofactor/ cosubstrate/vitamine binding	1.61E-04	**GPD1**, YDL124w, **SDH4**, SER3, **GND2**, **OYE2**, **MCR1**, **OYE3**
20.01.01	Ion transport	2.47E-03	PMP1, CCC2, SPF1, PIC2, CTR2, KHA1, **ATP14**, **ATP18**, MEP2, **ATP19**, **SSU1**
20.01.15	Electron transport	5.21E-10	**GRX1**, **STF1**, **SDH4**, **GRX2**, **CYC7**, **YEL047c**, **GRX4**, **STF2**, **OYE2**, **MCR1**, **ATP14**, **ATP18**, **ERO1**, **ATP19**, SIA1, **OYE3**

(continued)

Table 2 (continued)

MIPS ID	Functionary category	*p*-value	Entries
32.01	Stress response	1.48E-19	**SSA1**, **PRX1**, **SSA3**, **NTH2**, YRO2, TIP1, **HSP26**, **TPS1**, **SSE2**, **GRX1**, HSP30, **GPD1**, **NTH1**, **TPS2**, SED1, **HSP42**, **HSP78**, **GRX2**, **HSP31**, **CYC7**, PRB1, HOR2, **SSA4**, DDI1, **GRX4**, **HSP12**, **STF2**, **NQM1**, PAU13, **COS8**, **SOD2**, **GRE3**, HSP150, **LHS1**, **MCR1**, **HSP104**, UBI4, **AHP1**, **CRP6**, DAK1, **TSL1**, HOR7, **APJ1**, YGP1, DDR2, **GRE2**, GCY1, **SSE1**, **HSP82**, **HSP32**, **ATH1**
32.01.01	Oxidative stress response	1.84E-06	**PRX1**, **GRX1**, **GRX2**, **GRX4**, **HSP12**, **NQM1**, **SOD2**, **MCR1**, **AHP1**, **GRE2**
32.01.07	Unfolded protein response	2.75E-07	**SSA1**, **HSP26**, **HSP42**, **HSP78**, **HSP31**, **SSA4**, **COS8**, **LHS1**, **CRP6**, **APJ1**, **SSE1**, **HSP32**
32.07	Detoxification	1.61E-05	**PRX1**, **ADH5**, **GRX1**, SFA1, **GRX2**, **GRX4**, **NQM1**, **SOD2**, TPO1, **AHP1**, **GRE2**, SRL1, **SSU1**

Source: Based on data from Ogawa et al. (2000), Alexandre et al. (2001), Chandler et al. (2004), Marks et al. (2008), Dinh et al. (2009), and Ma and Liu (2010a). Proteins in bold indicate more than one function have been described

Chandler et al. 2004; Ma and Liu 2010a). Other cell wall related genes reported to be associated with ethanol tolerance by gene deletion mutant studies include *SMI1* in the regulation of cell wall synthesis, *ANP1*, *MNN10*, *MNN11*, and *HOC1* encoding the four subunits of mannosyltransferase complex, *LDB7* and *VMA9* involved in mannoprotein biosynthesis, *KRE6* encoding β-glucan synthase for β-1, 6-glucan biosynthesis, *WSC3*, *SLG1*, and *SLT2* encoding sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway involved in maintenance of cell wall integrity, and *MID2*, *ROM2*, *SIT4* related to cell wall organization (Kubota et al. 2004; Fujita et al. 2006; Van voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009).

Recently, 13 genes in PDR family were identified as candidate genes for ethanol tolerance (Ma and Liu 2010a). Among which *PDR1*, *PDR5*, *PDR12*, *YOR1*, *SNQ2*, *ICT1*, *DDI1*, *TPO1*, *GRE2*, and *YMR102C* displayed enriched background of transcription abundance, and *PDR15*, *DDI1*, *TPO1*, and *GRE2* maintained higher levels of transcription under ethanol stress over time in a tolerant strain. Many PDR genes function as transporters of ATP-binding cassette proteins and are encoded for plasma membrane proteins that mediate membrane translocation of ions and a wide range of substrates. It impacts lipid and cell wall compositions and major facilitator superfamily proteins for cell detoxifications (Jungwirth and Kuchler 2006; Gulshan and Moye-Rowley 2007). Since plasma membrane and cell wall are major targets

of ethanol damages, these PDR genes are hypothesized to be involved in reconditioning and remodeling membrane and cell walls in response to ethanol challenges (Ma and Liu 2010a).

3.1 Vacuole

Vacuole in yeast involves in numerous functional processes, including the homeostasis of cell pH and the concentration of ions, osmoregulation, storage of amino acids and polyphosphate, and degradation processes. Vacuole was demonstrated to be associated with ethanol tolerance. Deletion mutations of many genes related to vacuolar membrane structure and function, vacuolar protein sorting machinery, were sensitive to ethanol stress (Kubota et al. 2004; Fujita et al. 2006; van voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009) (Table 3). It is known that ethanol increases membrane permeability to protons causing increased proton influx and intracellular acidification (Cartwright et al. 1987; Rosa and Sá-Correia 1996). To counteract ethanol stress, transportation of intracellular H^+ to vacuoles by H^+ V-ATPase is important for yeast to maintain a sound intracellular pH homeostasis (Forgac 1998; Inoue et al. 2005). Most deletion mutants of genes encoding structural components of V-ATPase showed sensitive response to ethanol challenges, such as *VMA1*, *VMA2*, *VMA4*, *VMA5*, *VMA6*, *VMA7*, *VMA8*, *VMA9*, *VMA10*, *VMA11*, *VMA13*, *VMA16*, *VPH1*, and *CUP5* (Kubota et al. 2004; Fujita et al. 2006; van voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009) (Table 3). In addition, *VMA12*, *VMA21*, *VMA22*, *RAV1*, and *RAV2*, involved in the assembly of the V-ATPase, appeared to be associated with the tolerance. As anticipated, transportation of intracellular H^+ to the vacuole by H^+ V-ATPase caused vacuolar acidification in a dose-dependent pattern (Teixeira et al. 2009).

3.2 Mitochondrion

The mitochondrion is required for ATP regeneration as source of chemical energy and participates in the biosynthesis of phospholipids, degradation of fatty acids, amino acids and the storage of metal ions (Scheffler 1999). Under ethanol stress, genes encoding subunits of F-type ATP synthase (*ATP14*, *ATP18*, *ATP19*) in mitochondria were up-regulated (Dinh et al. 2009). Many ethanol-induced genes are located in mitochondria (Table 1). The ethanol-tolerant strains exhibited a lower frequency of ethanol-induced respiratory deficient than ethanol-sensitive strains (Chi and Arneborg 1999). This indicates that the ethanol tolerance of *S. cerevisiae* is dependent on the maintenance of functional mitochondria under the stress. Mitochondrial superoxide dismutases (Sod1p and Sod2p) are considered important for ethanol tolerance in *S. cerevisiae* in the post-diauxic phase (Costa et al. 1997).

Since mitochondrion structure was clearly observed at the end of brewing, it was suggested that it plays important roles during ethanol fermentation (Kitagaki and Shimoi 2007).

3.3 *Peroxisome*

Peroxisomes contain enzymes for certain oxidative reactions, such as beta-oxidation of very-long-chain fatty acids and many other metabolites. Peroxisomal function appeared to be important for ethanol tolerance in yeast. Strains with gene deletions of many genes, which encode proteins for peroxisomal transport machinery and peroxisomal membrane protein import machinery and for peroxisomal organization and biogenesis, were sensitive to ethanol challenge (Teixeira et al. 2009; Yoshikawa et al. 2009) (Table 3). However, all the deletion mutants of peroxisome targeting signaling (PTS2) receptor genes (such as *PEX7*, *PEX18*, and *PEX21*) were not sensitive to ethanol as well as genes regulating peroxisome size and numbers (such as *PEX11*, *PEX25*, *PEX27*, *PEX28*, *PEX29*, *PEX30*, *PEX31*, and *PEX32*). Furthermore, strains with deletion of genes encoding lysine biosynthesis and β-oxidation of fatty acids occurred in peroxisome were not sensitive to ethanol (Yoshikawa et al. 2009). Thereafter, peroxisome is hypothesized to play a role in synthesis or degradation of membrane phospholipids in cell membrane remodeling since cells deficient in peroxisomal functions are unable to effectively control fatty acid composition of membrane phospholipids (Lockshon et al. 2007). Another possible role of peroxisome is to metabolize peroxides and other reactive oxygen species (ROS), which are possibly imposed on yeast cells indirectly under ethanol stress (Du and Takagi 2007; Schrader and Fahimi 2004).

4 Amino Acid Encoding Genes

Amino acid biosynthesis is in general believed to be inhibited by ethanol. But recent studies indicated that enhanced expression of genes for biosynthesis or transportation of some amino acids increased ethanol tolerance. At least, tryptophan and proline have been demonstrated to have such function related to ethanol tolerance.

4.1 *Tryptophan*

Tryptophan is believed to be associated with ethanol tolerance. Deletion of genes *TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5* in any step of tryptophan biosynthesis

Table 3 Functional categories of genes whose deletion strains were sensitive to ethanol in *Saccharomyces cerevisiae*

MIPS functional category number	MIPS functional category	*p*-value	Entries
01.01.09.06	Metabolism of tryptophan	2.91E-05	TYR1 TRP1 TRP4 TRP2 TRP5 ARO1 ARO2 PRS3 TRP3 PRS5 ARO7
01.01.09.06.01	Biosynthesis of tryptophan	4.21E-06	TYR1 TRP1 TRP4 TRP2 TRP5 PRS3 TRP3 PRS5
01.05.25	Regulation of C-compound and carbohydrate metabolism	7.51E-07	RTG3 TPS1 CDC10 FEN2 REG1 NGG1 SNF1 MIG1 HAP2 RTG2 KRE11 SMI1 SNF6 PFK26 SWI3 GRR1 VPS25 HAP4 SNF7 ROM2 VPS36 PFK2 GLC8 SSN8 MKS1 RAS2c SIN4 POP2 RTG1 HAP5 SNF8 PHO85c GCR1 TAF14 BEM4
2.11	Electron transport and membrane-associated energy conservation	9.77E-03	ATP1 COX9 ATP5 QCR7 RIP1 QCR6 QCR9 RAV1 ATP7 COX12 NDE1 COQ10 ATP15 QCR2
02.13.03	Aerobic respiration	3.35E-09	PET112 ETR1 COX9 PET100 YDR115w RSM24 BCS1 QCR7 RIP1 QCR6 SHY1 QCR9 DIA4 COX23 COX16 CBP1 OAR1 COX12 FMP53 COQ5c COX14 NDE1 MRPS17 PPA2 HER2 POR1 MRPL22 COX11 QCR2
10.03.01.01	Mitotic cell cycle	5.12E-03	CLN3 SPC72 PIN4 BIK1 SIT4 SWM1 SWI4 CDH1 CKB1 DOC1 ARP1 IRR1 SWE1 PTK2 GRR1 SAP190 SWI6 RSC2 CIK1 MCK1 CSE2 TRF4 CKB2 BFR1 TAF14 KIP2 NIP100 CTF4 KAR3
11.02.03.01	General transcription activities	1.42E-04	RRN10 RTG3 CYC8 TFC1 MED8 PAF1 MED2 TFB5 DPB4 HPR1 NGG1 REF2 SPT3 HAC1 PGD1 MIG1 DST1 RPB9 SOH1 RTF1 SRB5 ELP2 STP2 SRB2 THP2 CST6 MET18 RPB4 BYE1 SWI6 IKI3 MFT1 MAC1 MTF1 ELP6 SSN8 BDP1 THO2 SKO1 SIN4 CSE2 RTG1 CTR9 SPT20 LEO1 MBF1 TAF14
11.02.03.01.04	Transcription elongation	2.01E-05	PAF1 HPR1 DST1 RTF1 ELP2 THP2 BYE1 IKI3 MFT1 ELP6 THO2 CTR9 LEO1
11.02.03.04	Transcriptional control	9.16E-04	DEP1 RTG3 SPT7 CYC8 MED8 PAF1 MED2 MBP1 MAF1 KCS1 REG1 TFB5 HPR1 ARG82 NGG1 UME6 HDA2 SPT3 GCN4 SWI4 GLO3 CAF16 HAC1 PTR3 PGD1

(continued)

Table 3 (continued)

MIPS functional category number	MIPS functional category	*p*-value	Entries
			MIG1 AFT1 HAP2 RTG2 DBF2 SRB5 ELP2 GCN5 YAP3 SNF6 STP2 SRB2 SKN7 CST6 MET18 CTK2c SPT10 TPK1 SWI3 VPS25 BYE1 HAP4 RIC1 SWI6 IKI3 BDF1 SFP1 VPS36 CTK3 SOK2 MAC1 ELP6 HDA1 SSN8 MKS1 YAF9 EAF7 SKO1 SIN4 CAF40 CSE2 POP2 PHO80 SIN3 RTG1 SPT20 MBF1 HAP5 SNF8 PHO85c GCR1 TAF14 CTI6 EAF3 NOT5 FHL1
12.01.01	Ribosomal proteins	4.32E-04	RPL19B RPS8A MRP21 MRPS5 MRPL37 MRPL27 MRPL32 IMG1 IMG2 RPP1A RPL35A YDR115w RSM24 MRPL7 MRPS28 MRP1 RSM18 RPS26B RSM23 RPL1B RPS25A MRPL25 RPL11B MRPL6 RPL34B MRPL49 RPS4A RSM22 MRP49 MRPL38 MRPL13 DBP7 MRPL20 RPL13B MRPS8 MRPS17 MRPL33 SWS2 MRPL22 MRPS12 RSM19 PET123 RPL20B MRPS16 RPS6A MRPL40 MRP2
14.04	Protein targeting, sorting, and translocation	3.02E-11	PEX22 NUP170 VPS15 CCZ1 SEC66 SSH1 STP22 PEX19 ASM4 NUP84 MAF1 NUP42 PEX5 PEX3 VPS74 VPS72 CUP5 KAP123 FAB1 MON1 PEX14 PEX8 TIM13 VPS29 SEC28 VPS35 MOG1 VPS25 DID4 VPS24 NUP120 NUP100 LHS1 DID2 NUP133 ATG10 SNF7 SRN2 PEP3 LIP2 VPS38 VPS33 VPS36 VPS71 MFT1 VPS9 MVP1 IMP2 SAM37 IMP1 TOM40 PEP5 YDJ1 TOM7 TOM70 MON2 VPS27 COQ10 PEX15 RTG1 VPS68 PEP12 VPS5 VPH1 SNF8 VPS16 VPS28 VPS30 ATG11 TOM5 VPS66 VPS4
14.07.04	Modification by acetylation, deacetylation	7.68E-04	SPT7 SGF29 NAT1 NGG1 HDA2 SPT3 SGF73 NAT2 GCN5 ARD1 SPT10 IKI3 HDA1 EAF7 SIN3 SPT20 EAF3 NAT3

(continued)

Table 3 (continued)

MIPS functional category number	MIPS functional category	*p*-value	Entries
14.10	Assembly of protein complexes	1.28E-09	CYC3 SLA1 PIM1 SCO1 TCM62 SPT7 RAD18 PRP11 COX9 MSS2 ARF1 PET100 VPS41 NBP2 PEX10 BCS1 QCR7 PET117 RAD6 COX18 VMA21 PEX4 CBP4 VMA22 FMC1 COX16 ATP12 GRR1 ATP7 VMA5 CYT2 VPH2 PEX1 COX19 COX12 ACF2 ATP10 VMA6 COX14 SAM37 YTA12 END3 BNI4 SLA2 ATP11 VAM3 VPH1 VMA4 COX11 YPL172c TFP3 YME1 KAR3 QCR2
16.01	Protein binding	4.79E-04	SLA1 PIN4 TCM62 HSP26 UMP1 MED8 BEM1 STP22 BIK1 RVS161 RAD18 DHH1 GCS1 PET100 SAC6 MFB1 PEX5 PEX10 SNF1 VPS52 GIM4 BMH1 MDJ1 RAD6 PEX14 PAC10 SHY1 PEX4 BUB1 VMA22 IRR1 CAP2 PFD1 ATP12 RCY1 PEX2 MOG1 GRR1 DID4 LHS1 PEP3 SWI6 YKE2 BUD6 VRP1 SSQ1 GIM5 PEX12 SAM37 AIP1 HSC82 LST8 SIS1 TPM1 END3 SRV2 PEX17 BNI4 SLA2 BNI1 ATP11 VPS27 SLG1 SHE4 RBL2 CIN1 NIP100 CTI6
16.07	Structural protein binding	4.39E-03	NUP170 SPT7 ASM4 NUP84 NUP42 ATP5 CLC1 ATP7 NUP120 NUP100 NUP133 BUD6 BNI1 ARC35
18.02	Regulation of protein activity	3.03E-03	CLN3 CSG2 FES1 CYC8 CCZ1 UMP1 STE50 VAM6 GCS1 REG1 VPS41 NGG1 SPT3 SNF1 GLO3 BEM2 PTR3 CDH1 DOC1 RRD1 CTK2c SWE1 KTI12 RIC1 SWI6 ROM2 VAC14 VPS36 VPS9 MAC1 MTF1 GLC8 SSN8 YDJ1 MCK1 SEC12 PHO80 RTG1 SPT20 WHI2 GYP1 RGA1 MBF1
20.01.01.01	Cation transport (H^+, Na^+, K^+, Ca^{2+}, NH^{4+}, etc.)	1.33E-04	DRS2 ATP1 SCO1 VMA2 GGC1 VPS41 ATP5 CUP5 SPF1 VMA8 FTR1 AFT1 VMA7 PPA1 VMA10 ATP7 VMA5 COX19 ISA1 NHA1 VMA6 CCS1 VPH1 VMA4 TFP3 ATP15 VMA13 ISA2 CTR1

(continued)

Table 3 (continued)

MIPS functional category number	MIPS functional category	*p*-value	Entries
20.01.15	Electron transport	1.19E-03	ATP1 VMA2 COX9 ATP5 CUP5 VMA8 ARO2 VMA7 PPA1 VMA10 ATP7 VMA5 COX12 VMA6 NDE1 VPH1 VMA4 TFP3 ATP15 VMA13
20.03.22	Transport ATPases	1.29E-06	DRS2 ATP1 VMA2 VMA9 ATP5 BCS1 CUP5 SPF1 VMA8 VMA7 PPA1 VMA10 ATP7 VMA5 VMA6 VPH1 VMA4 TFP3 ATP15 VMA13
20.09.03	Peroxisomal transport	1.27E-04	PEX22 PEX19 PEX5 PEX3 PEX14 PEX8 PEX2 PEX17 PEX15
20.09.07	Vesicular transport (Golgi network, etc.)	4.42E-04	DRS2 VPS15 SED4 ARF1 GCS1 VPS54 GSG1 VPS52 GLO3 ERV14 VAM7 KRE11 CLC1 ERV29 VPS29 SEC28 APS3 VPS35 DID4 PEP3 SUR4 VPS33 VPS36 YPT7 VPS9 PEP5 LST8 COG5 YDJ1 VPS27 SEC12 PEP12 VPS5 GYP1 VAM3 TRS33 RUD3 VPS16 VPS30 VPS4
20.09.13	Vacuolar transport	1.67E-16	VPS15 VMA2 CCZ1 STP22 FEN1 VPS74 VPS72 CUP5 VMA8 FAB1 PIB2 MON1 VAM7 VMA7 CLC1 VPS29 VMA10 VPS35 VPS25 DID4 VPS24 VMA5 DID2 ATG10 SNF7 SRN2 PEP3 LIP2 VPS38 VPS33 VPS36 VMA6 YPT7 VPS71 VPS9 MVP1 VPS20 PEP5 TPM1 MON2 VPS27 VPS68 PEP12 GYP1 VAM3 VPH1 VMA4 SNF8 VPS16 VPS28 BRO1 VPS30 TFP3 VMA13 ATG11 VPS66 VPS4
30.01.05.05.01	Small GTPase mediated signal transduction	2.27E-03	BOI2 BEM2 BMH1 TPK1 RHO4 BUD6 ROM2 RAS2c SRV2 BNI1 CLA4 WSC3 SLG1 RGA1 BEM4
34.01.01.03	Homeostasis of protons	5.22E-10	ATP1 VMA2 VMA9 RAV2 ATP5 CUP5 VMA8 VMA7 PPA1 VMA10 VMA22 RAV1 ATP7 VMA5 VPH2 MEH1 NHA1 VMA6 VPH1 VMA4 TFP3 ATP15 VMA13

(continued)

Table 3 (continued)

MIPS functional category number	MIPS functional category	*p*-value	Entries
40.01	Cell growth/ morphogenesis	4.76E-06	TPD3 CLN3 SLA1 BEM1 STE50 CDC10 RVS161 FEN1 SHS1 REG1 EXG2 SSD1 SNF1 PAC11 BOI2 BMH1 CDH1 CKB1 CLC1 PRS3 SLT2 HTD2 ARP1 SKN7 CAP2 BCK1 SWE1 HOC1 GRR1 ELM1 KTI12 RHO4 ACF2 MID2 VRP1 ROM2 SFP1 AIP1 TPM1 SRV2 WHI3 BNI4 SLA2 BNI1 CLA4 ARC35 WHI2 RGA1 BEM4 NIP100 PLC1 KRE6
42.04.03	Actin cytoskeleton	8.05E-05	TPD3 SLA1 SIT4 VPS54 SAC6 MNN10 VPS52 BEM2 CAP2 RHO4 ACF2 BUD6 VRP1 ROM2 AIP1 TPM1 END3 SLA2 BNI1 WSC3 SLG1 SHE4 WHI2 RGA1 BEM4
42.16	Mitochondrion	5.54E-13	MDM10 PET112 MRP21 FZO1 MRPS5 MRPL37 MRPL27 CTP1 MRPL32 IMG1 IMG2 GGC1 YDR115w RSM24 MSS116 MRPL7 MRPS28 MRP1 BCS1 SHE9 UGO1 RSM18 GET1 RSM23 MRM2 MDM34 MRPL25 MRPL6 MRPL49 YJR120w RSM22 MRP49 MRPL38 MRPL13 MRPL20 MMM1 MDM30 SAM37 ABF2 NDE1 MRPS8 MRPS17 MTF1 MRPL33 POR1 MRPL22 ATP11 MRPS12 RSM19 MDM12 PET123 MRPS16 MRPL40 YME1 MRP2
42.19	Peroxisome	4.08E-04	PEX19 PEX5 PEX10 PEX3 PEX8 PEX4 PEX2 PEX1 PEX12 PEX17 PEX15 ATG11
42.25	Vacuole or lysosome	1.96E-05	CLN3 CCZ1 VAM6 KCS1 DOA4 VPS41 RAV2 CUP5 FAB1 VAM7 VPS29 PEP3 VPS33 PEP5 VPS16 VPS4
42.29	Bud/growth tip	5.95E-03	TPD3 BEM2 ELM1 ROM2 TPM1 BNI1 CLA4 RGA1 BFR1
43.01.03.05	Budding, cell polarity, and filament formation	9.26E-07	TPD3 CLN3 SLA1 SHP1 RXT2 BEM1 STE50 CDC10 RVS161 FEN1 BUD31 SIT4 SHS1 SAC6 MNN10 SWM1 SSD1 SPT3 SNF1 PAC11 BUD16 BOI2 BEM2 BMH1 BUD27 CKB1 ERV14

(continued)

Table 3 (continued)

MIPS functional category number	MIPS functional category	*p*-value	Entries
			DIA4 SLT2 ARP1 CAP2 BCK1 TPK1 SWE1 RCY1 HOC1 GRR1 ELM1 SAP190 RHO4 ACF2 BUD6 VRP1 ROM2 SUR4 SOK2 RIM9 AIP1 TPM1 END3 RAS2c SRV2 WHI3 BNI4 SLA2 BNI1 CLA4 SLG1 CKB2 RGA1 BFR1 RIM20 BEM4 NIP100 PLC1 AXL1
43.01.03.09	Development of asco- basidio- or zygospore	3.28E-03	SPO7 SHP1 ECM33 CDC10 FEN1 HEX3 NUP84 DOA4 GSG1 ARG82 SWM1 EXG2 SPT3 SPS1 BMH1 ERV14 RAD6 MDS3 NEM1 IRR1 AYR1 NUP133 SNF7 BDF1 RIM9 RAS2c CNM67 SIN4 MCK1 POP2 SIN3 RIM20

Source: Data from Kubota et al. (2004), Fujita et al. (2006), Van voorst et al. (2006), Auesukaree et al. (2009), Teixeira et al. (2009), and Yoshikawa et al. (2009). Function of gene products was classified using Functional Catalogue (FunCat) described in the Munich Information Center for Protein Sequences (MIPS) database (http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html)

resulted in sensitive response to ethanol stress (Kubota et al. 2004; Fujita et al. 2006; Hirasawa et al. 2007; Yoshikawa et al. 2009) (Table 3). Moreover, deletion of *PRS3*, *PRS5*, *ARO1*, or *ARO2* related to biosynthesis of tryptophan's precursor also displayed sensitive phenotype to ethanol (Kubota et al. 2004; Teixeira et al. 2009; Yoshikawa et al. 2009). Higher ethanol-tolerant brewing yeast showed higher expression levels for tryptophan biosynthesis genes (Hirasawa et al. 2007). All five tryptophan biosynthesis genes were less repressed over time for a tolerant yeast under ethanol stress compared with its parental strain (Ma and Liu 2010a). Overexpression of either tryptophan biosynthesis genes (*TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5*) or tryptophan permease gene (*TAT2*) improved ethanol tolerance, especially for *TRP2* and *TRP5*. In the meantime, supplementation of tryptophan to culture medium enhanced yeast tolerant levels to ethanol (Hirasawa et al. 2007).

4.2 Proline

In yeast, proline has multiple functions during fermentation process, including protection of cells from damage by freezing, desiccation, or oxidative stress (Takagi 2008). It enhances stability of proteins and membranes, and inhibits protein aggregation during protein refolding (Rudolph and Crowe 1985; Samuel et al. 2000).

When the wild-type *PRO1* gene was replaced by $pro1^{D154N}$ allele, the yeast showed increased biosynthesis of proline and improved tolerance to ethanol stress (Takagi et al. 2005). Function of proline in ethanol tolerance was also supported by a *PRO1*-deletion strain, which was more sensitive to ethanol stress (Kubota et al. 2004; Yoshikawa et al. 2009). Under ethanol stress, expressions of *PRO1, PRO2*, and *PRO3* for de novo biosynthesis of proline were not significantly induced (Kaino and Takagi 2008; Ma and Liu 2010a). Up-regulated expression of *PUT4* encoding a high-affinity proline transporter was demonstrated to contribute to the accumulation of proline in yeast cells (Kaino and Takagi 2008). This suggests the accumulation of proline is caused by import of proline from medium but not by de novo biosynthesis. Over accumulation of intracellular proline is often associated with reduced growth rate in *S. cerevisiae* and delayed yeast cell growth in the presence of ethanol (Maggio et al. 2002; Takagi et al. 2007). The amount of intracellular proline appeared to be maintained at well-controlled levels in order to cope with ethanol stress and a delayed growth.

5 Heat Shock Proteins

Ethanol stress damages protein conformation and causes aggregation of denatured proteins. Heat shock proteins, mainly acting as chaperones, are commonly induced under ethanol stress for protecting proteins as well as cell structure and organelles.

5.1 Chaperones

Under ethanol stress condition, induced expression of heat shock protein genes is commonly observed. At least 10 HSP genes *HSP12*, *HSP26*, *HSP30*, *HSP31*, *HSP32*, *HSP42*, *HSP78*, *HSP82*, *HSP104*, and *HSP150* were identified as up-regulated (Piper et al. 1994; Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Marks et al. 2008; Ma and Liu 2010a). The ethanol-induced expression can be concentration-dependent or strain-dependent. For example, transcript of *HSP26* was undetectable with an addition of 2% ethanol, barely detectable by 4% ethanol, and much abundance with further increased ethanol concentrations (Piper et al. 1994). Induction of HSP genes can be detected in both ethanol-tolerant and its parental strains under ethanol stress. However, a lack of continued function of a gene can lead to no metabolic functions for a sensitive strain under pressure. As observed for tolerant strain Y-50316, HSP genes *HSP12, HSP32, HSP42, HSP78, HSP82*, and *HSP150* were constitutively expressed over time under ethanol stress that allowed yeast to establish a viable culture under 8% ethanol challenge (Fig. 1) (Ma and Liu 2010a). Such dynamic expression event contributes to a meaningful tolerance phenotype.

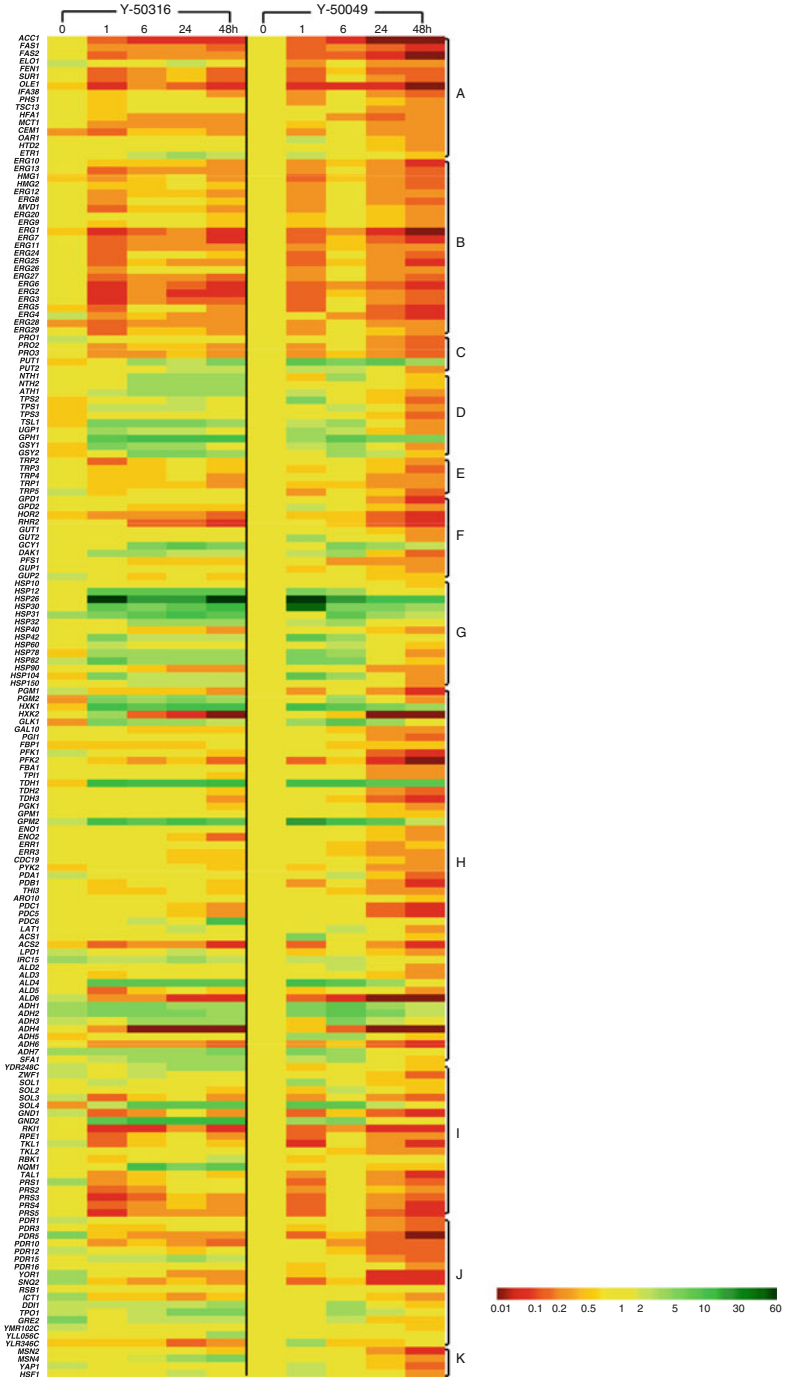

Fig. 1 Comparison of mRNA expression of *Saccharomyces cerevisiae* ethanol- and inhibitor-tolerant mutant NRRL Y-50316 and its parental strain NRRL Y-50049 by fold changes from 0 to

HSPs, mainly acting as chaperones, insure proper folding or refolding of other nascent or denatured proteins and enzymes to maintain a functional conformation (Parsell et al. 1994; Young et al. 2004; McClellan et al. 2007; Gong et al. 2009). Some HSPs are also involved in disassembling aggregates of misfolded proteins, such as Hsp104p, Hsp70p, and Hsp40p (Glover and Lindquist 1998). Besides HSP genes, other genes encoding chaperones, such as *SSA1*, *SSA2*, *SSA3*, *SSA4*, *SSE1*, *SSE2*, *APJ1*, and *LHS1,* involved in protein folding and refolding are also highly up-regulated under ethanol stress (Alexandre et al. 2001; Chandler et al. 2004; Marks et al. 2008). Interactions between different chaperones existed widely, which imply correct folding or stability of certain proteins may need more than two different chaperones in participation for efficient functions (Gong et al. 2009), or protein folding at different stage may need different chaperones (Young et al. 2004). Therefore, induction of multiple chaperones may be necessary to counteract ethanol stress. At the same time, certain functional chaperones are required for the folding of more difficult-to-fold proteins from nascent polypeptides into biologically active structures as well as for the refolding of denatured proteins back into native conformations. For example, *HSP82* displayed high transcription abundance (Ma and Liu 2010a), and its encoding protein Hsp82p has been reported to activate many key proteins such as transcription factors and regulatory kinases (Picard 2002; Prodromou and Pearl 2003; Young et al. 2004; McClellan et al. 2007). Since chaperones are widely spread in locations of cytoplasm, nucleus, mitochondria, membrane, and others (Table 1), interactions with many genes at multiple loci over time may be important for cell functions under the stress (Fig. 2). Since ethanol perturbs protein conformation and causes accumulation of denatured proteins, protein repairing functions over time by multiple chaperones appear to be critical for yeast tolerance to ethanol (Fig. 2).

5.2 Other Functions

Hsp150p was identified as a protein for cell wall stability and remodeling (Moukadiri and Zueco 2001). It is secreted and covalently attached to cell wall via beta-1,3-glucan and disulfide bridges. *HSP12*, encoding a plasma membrane, was highly induced over time in ethanol-tolerant strain (Ma and Liu 2010a). It was

Fig. 1 (continued) 48 h after the ethanol challenge treatment as examined by real time qRT-PCR array assays. Corresponding genes were categorized by functions involved in fatty acid biosynthesis (**a**), ergosterol metabolism (**b**), proline metabolism (**c**), trehalose metabolism (**d**), tryptophan metabolism (**e**), glycerol metabolism (**f**), heat shock protein family (**g**), glycolysis (**h**), pentose phosphate pathway (**i**), pleiotropic drug resistance gene family (**j**), and related transcription factor genes (**k**). Expression for a gene at each time point was presented in relative fold changes against that of Y-50049 at 0 h. *Green* indicates enhanced expression, *red* for repressed expression, and *yellow* for no significant changes. Scales of expressions were indicated by an *integrated color bar* at the *bottom*

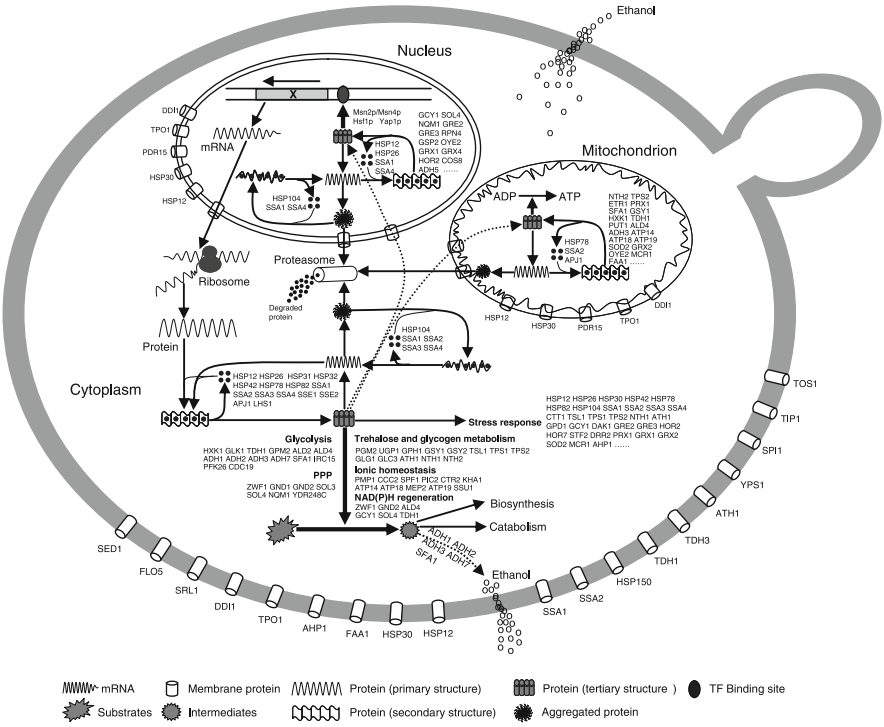

Fig. 2 A schematic diagram showing a prototype of mechanisms for ethanol tolerance in *Saccharomyces cerevisiae*. Proteins encoded by significantly up-regulated genes are located in cell wall, membrane, nucleus, mitochondrion, and cytoplasm. Heat shock proteins are mainly detailed as chaperones protecting and maintaining proteins functions at multiple loci. Functions of gene products are classified based on Gene Ontology. Figure legends are provided under the illustration. This figure is based on Ma and Liu (2010a) with modifications using data from Ogawa et al. (2000), Alexandre et al. (2001), Chandler et al. (2004), Marks et al. (2008), and Dinh et al. (2009)

demonstrated to be responsible for increased integrity on the liposomal membrane in the presence of ethanol, and yeast strains unable to express Hsp12p were sensitive to ethanol (Sales et al. 2000). Chaperones Ssa1p and Ssa2p were found to localize to cell walls in addition to nucleus and cytoplasm (López-Ribot and Chaffin 1996). Hsp30p, a hydrophobic plasma membrane protein, was reported as a negative regulator of H^+-ATPase Pma1p (Piper et al. 1997). Activity of plasma membrane H^+-ATPase consumes ATP as energy to pump proton. Under ethanol stress, ATP generation from glycolysis is inhibited. Therefore, induction of *HSP30* might provide an energy conservation role, limiting excessive ATP consumption by plasma membrane. But greatly induction of *HSP30* may lead to intracellular acidification in yeast cells that disrupts pH and ionic homeostasis, causing cells enter into cell cycle arrest (Ma and Liu 2010a). Except function as chaperones, Hsp31p and Hsp32p have functions of hydrolase activity and peptidase activity to degrade unrecoverable proteins (Wilson et al. 2004).

6 Pathway Analysis

Glucose metabolic pathway provides ATP as energy and intermediate metabolites for biosynthesis that is vital for cell functions. Three major glucose metabolic pathways, including trehalose and glycogen metabolisms, glycolysis and fermentation, and pentose phosphate pathway are discussed in this section.

6.1 Trehalose and Glycogen Metabolisms

Trehalose accumulation was observed in yeast cells, and cells unable to accumulate trehalose displayed retarded growth under ethanol challenges (Mansure et al. 1994; Ogawa et al. 2000; Kaino and Takagi 2008). Trehalose has been reported to function by reducing membrane permeability as well as ensuring proper folding of proteins (Mansure et al. 1994; Singer and Lindquist 1998). (See Chap. 2 in this volume for detailed discussions on trehalose metabolism.) Under ethanol stress conditions, up-regulated expression of genes involved in trehalose synthesis, including *TPS1*, *TPS2*, *TSL1*, *PGM2*, and *UGP1*, was generally observed (Fig. 3) (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a). The intermediate trehalose-6-phosphate is a regulator of yeast glycolysis that inhibits hexokinase (Blázquez et al. 1993). Such inhibition avoids depletion of intracellular Pi and ATP by over phosphorylation of glucose (François and Parrou 2001). Genes involved in trehalose degradation, including *NTH1*, *NTH2*, and *ATH1*, were also induced by ethanol (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a). Enhanced expression of trehalose degradation genes appeared to be required for balancing trehalose concentration and avoid side effect to others enzymes, such as glutathione reductase, cytosolic pyrophosphatase, and glucose 6-phosphate dehydrogenase (Sebollela et al. 2004).

Glycogen metabolism is very close to trehalose pathway and displays very similar expression pattern with trehalose metabolism (Fig. 3). Genes involved in both glycogen biosynthesis (*GSY1* and *GSY2*) and degradation (*GPH1*) were induced by ethanol (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a). Whether variations of intracellular glycogen concentrations are related to increased ethanol tolerance remains to be confirmed. The futile energetic cycles of trehalose and glycogen are thought to facilitate the balance of ATP and Pi in yeast cell (Alexandre et al. 2001). Taken the closely related trehalose and glycogen metabolism into consideration, trehalose concentration could be subtly affected by glycogen metabolism, and the induced gene expression related to both trehalose biosynthesis and degradation may facilitate a stable intracellular environment for cell survival under ethanol stress.

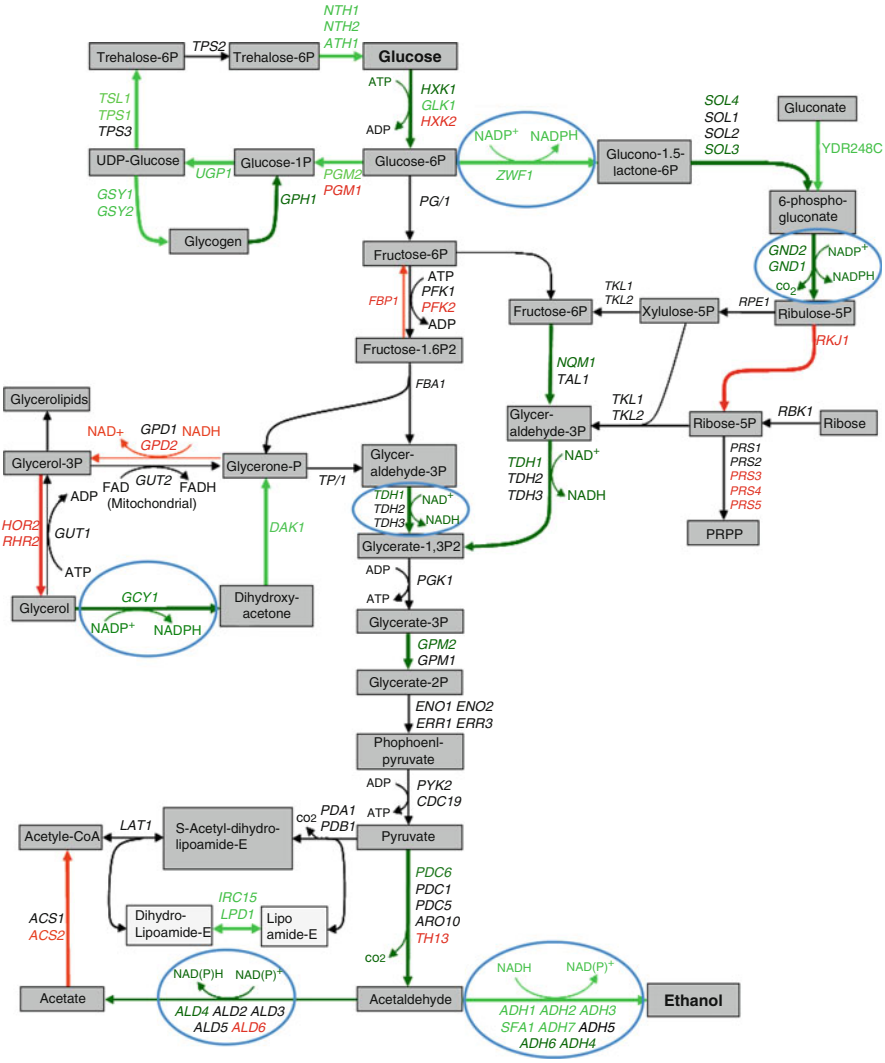

Fig. 3 Illustrative pathways of *Saccharomyces cerevisiae* ethanol- and inhibitor-tolerant mutant NRRL Y-50316 involved in trehalose-glycolysis-pentose phosphate pathway in response to ethanol challenges inferred by dynamic quantitative mRNA expression analysis and metabolic profiling analysis compared with its parental strain NRRL Y-50049. *Dark green arrowed lines and letters* indicate high levels (5.1- to 60-fold increase for at least one critical time point) of mRNA expression and enhanced pathways, *green* for significant levels (1.5- to 5-fold increase for at least one critical time point) of enhanced transcription and pathways; *black* indicates normal or nearly normal levels of transcription and pathway events, *red* for repressed expression, reactions, or pathways. *Bold lines and letters* indicate the levels of expression, and pathways are statistically significant at $P \leq 0.05$. Reactions involved in NAD(P)H regeneration steps are circled in *blue*

6.2 Glycolysis and Fermentation

Glycolysis plays an important role in cell growth and subsequent ethanol fermentation. It not only provides ATP as energy but also produces a variety of carbon intermediate metabolites for nucleotide, amino acid, and lipid biosynthesis. Transcriptional response of genes in glycolysis and fermentation pathway under ethanol stress was reported by using microarray with snapshot (Alexandre et al. 2001; Chandler et al. 2004). Using robust mRNA references, transcriptome response over time was quantitatively analyzed (Ma and Liu 2010a). *HXK1* and *GLK1* encoding hexokinase and glucokinase, respectively, and catalyzing the first step of glucose metabolism by phosphorylation, were up-regulated over time under ethanol stress (Fig. 3). *HXK1* showed higher transcription abundance than *GLK1* over time, indicating its potential important role for glucose phosphorylation. *TDH1* displayed about 20-fold increase of transcription level, the highest up-regulated gene in glycolysis and fermentation pathway (Ma and Liu 2010a). Tdh1p catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate, at the same time produces NADH. *GPM2* was another highly up-regulated gene, which encodes enzyme for conversion of glycerate-3P to glycerate-2P in glycolysis. Genes (*GPD2*, *HOR2*, and *RHR2*) encoding enzyme for glycerol biosynthesis were downregulated, but *GCY1* and *DAK1* for glycerol catabolism were up-regulated. *ALD4*, encoding major mitochondrial aldehyde dehydrogenase, is the only up-regulated gene for acetate conversion. As for genes encoding alcohol dehydrogenase, *ADH1*, *ADH2*, *ADH3*, *ADH7*, and *SFA1* were up-regulated over time. This indicates transcription of these genes is less sensitive to ethanol. In glycolysis and fermentation pathway, many important genes displayed normal or near normal transcription under 8% ethanol challenges for ethanol-tolerant strain, such as *PGK1*, *PYK2*, and *CDC19* for ATP regeneration. The enhanced expression of alcohol dehydrogenase genes *ADH1*, *ADH2*, *ADH3*, *ADH7*, and *SFA1*, together with other up-regulated, normal or near normal expression of genes in the intermediate steps of glycolysis are necessary to complete ethanol fermentation.

6.3 Pentose Phosphate Pathway

There are two distinct phases in the pentose phosphate pathway. The first is the oxidative phase, in which NADPH is regenerated, and the second is the non-oxidative biosynthesis of 5-carbon sugars for the biosynthesis of the nucleotides and amino acids. In oxidative phase, *ZWF1*, *SOL4*, *GND2*, and *YDR248C* (putative gluconokinase function) were up-regulated under ethanol stress (Ma and Liu 2010a) (Fig. 3). *GND2* was the highest up-regulated gene in the pentose phosphate pathway. In the non-oxidative phase, genes (*RPE1*, *TKL1*, *TKL2*, and *TAL2*) linking pentose phosphate pathway to glycolysis were normally expressed, and *NQM1*

encoding putative transaldolase was up-regulated. However, genes (*RKI1*, *PRS3*, *PRS4*, and *PRS5*) for the biosynthesis of nucleotides and amino acids were significantly repressed and reflected by slowing down the cell growth.

7 Cofactor Homeostasis and Ion Transport

Up-regulated expression of genes involved in regeneration of cofactors such as NADH and NADPH suggested their association with ethanol tolerance. Some genes involved in ion transport and homeostasis are induced by ethanol. Supplementation of minerals or trace minerals also improved ethanol tolerance.

7.1 Cofactor Redox Balance

Redox metabolism, in the form of interconversion of the pyrimidine nucleotide cofactors NAD^+/NADH and $NADP^+$/NADPH, plays important roles in yeast metabolism of amino acids, lipids, and nucleotides (Bruinenberg et al. 1983; Hou et al. 2009). Under ethanol stress, *ZWF1* and *GND2* related to NADPH regeneration in pentose phosphate pathway and *TDH1* related to NADH regeneration in glycolysis pathway were up-regulated (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a). Enhanced expression of *ZWF1*, *SOL4*, and *YDR248C* may provide sufficient substrate and accelerate downstream decarboxylation reactions to regenerate more NADPH by *GND2*. *DAK1* may assist NADPH regeneration by *GCY1* through a smooth flow of glycerol to glycerone-P. Similarly, enhanced expression of *NQM1* could accelerate a smooth flow of fructose-6P to glycerate-1,3P2 for more NADH regeneration by *TDH1*. Expression level of *GND2* and *TDH1* is observed always higher for tolerant strain than a wild type (Ma and Liu 2010a). Sufficient supply of NADH and NADPH in the reducing form likely contributes to ethanol tolerance indirectly through efficient biosynthesis of lipids, amino acid, and nucleotides for cell growth and viability (Martin et al. 2007).

7.2 Ion Transport

Optimized concentrations of minerals or trace minerals, such as calcium, magnesium, and zinc, are helpful to improve ethanol fermentation rate and final ethanol concentration (Nabais et al. 1988; Birch and Walker 2000; Zhao et al. 2009). Ethanol affects the translocation of ions such as Ca^{2+} and Mg^{2+} (Dombek and Ingram 1986; Walker and Maynard 1997). *PMP1*, *CCC2*, *SPF1*, *PIC2*, *CTR2*, *KHA1*, *MEP2*, *SSU1*, *ATP14*, *ATP18*, and *ATP19* were observed to be induced under ethanol stress (Alexandre et al. 2001; Chandler et al. 2004; Dinh et al. 2009)

(Table 2), which are involved in copper, iron, calcium, potassium, phosphate, ammonium, and sulfur ionic homeostasis (Marini et al. 1997; Yuan et al. 1997; Park and Bakalinsky 2000; Portnoy et al. 2001; Cronin et al. 2002; Hamel et al. 2004). Deletion mutations demonstrated at least 29 genes related to cation transport (H^+, Na^+, K^+, Ca^{2+} , $NH4^+$, etc.) displayed sensitive to ethanol stress (Table 3). Maintenance of ionic homeostasis is related to H^+-ATPase, and maintenance of the electrochemical proton gradient by the H^+-ATPase is vital for ion exchange under ethanol stress (Ramírez et al. 1998). Up-regulated expression of *ATP14*, *ATP18*, and *ATP19* might contribute to the maintenance of both pH and ionic homeostasis.

8 Regulatory Networks

Yeast response to ethanol stress is triggered via a complicated signal transduction pathway. The activated signal transduction pathway by ethanol activates stress transcription factors such as Msn2p/Msn4p, Yap1p, and Hsf1p. As a result, the transcription factors enter into nucleus and bind to stress response elements in promoter regions of target genes to induce their expression.

8.1 Signal Transduction Pathways

Ethanol as a general stress factor for yeast triggers the main signal transduction pathway for stress response through activating Msn2p/Msn4p. The first signal transduction pathway implicated in activation of Msn2p/Msn4p is cAMP-protein kinase pathway. In this signal transduction pathway, the second messenger cAMP is synthesized by adenylate cyclase encoded by *CYR1*, which can be activated either by G protein-coupled receptor system Gpr1p-Gpa2p or Ras1p/2p (Colombo et al. 1998; Thevelein and de Winde 1999; Estruch 2000; Thevelein et al. 2000; Costa and Moradas-ferreira 2001; Müller et al. 2003; Nikolaou et al. 2009). Under normal physiological conditions, glucose triggers G protein-coupled receptor system to activate adenylate cyclase for higher levels of cAMP generation, and cAMP activates protein kinase A (PKA) for cell growth. At the same time, activated PKA inhibits Msn2p/Msn4p for general stress response as well as Yap1p and Skn7p for specific stress responses (Fig. 4). Similarly, members of HSP70 (such as Ssa1p and Ssa2p) and/or HSP90 (such as Hsp82p) protein family interact with Cdc25p to regulate Ras1p/2p and cAMP-PKA pathway (Geymonat et al. 1998). This pathway plays a critical role in adaptation of cells to stress conditions. Under stress conditions, HSPs are recruited to participate in refolding proteins to maintain their native conformation for function (Young et al. 2004; McClellan et al. 2007; Gong et al. 2009). This process reduces interactions of HSPs with Cdc25p/Sdc25p and thereafter decreases signal transduction involved in cAMP-PKA pathway (Thevelein and de Winde 1999). Msn2p and Msn4p contain a nuclear localization

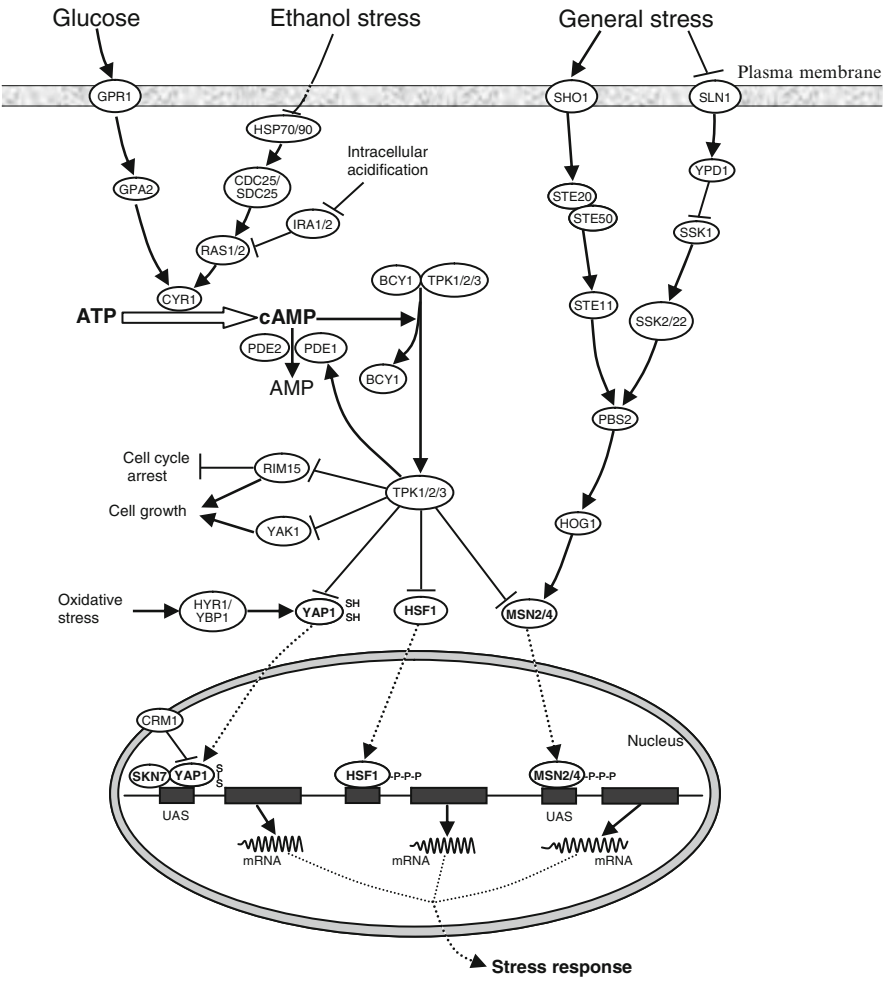

Fig. 4 A schematic diagram showing signal transduction pathways involved in ethanol-tolerant stress response in *Saccharomyces cerevisiae* with transcription factors in bold. *A line ended with an arrow* indicates a positive interaction, and with *a bar*, a negative interaction (This figure is adapted based on Colombo et al. (1998), Geymonat et al. (1998), Thevelein and de Winde (1999), Estruch (2000), Thevelein et al. (2000), Costa and Moradas-Ferreira (2001), Hohmann (2002), Müller et al. (2003), Ferguson et al. (2005), and Nikolaou et al. (2009))

signal (NLS) inhibited by PKA phosphorylation (Görner et al. 1998, 2002). Downregulation of cAMP-PKA pathway releases this inhibition; causes transfer of Msn2p/Msn4p from cytoplasm to nucleus and further hyperphosphorylated by other stress-activated kinases to trigger the stress responses (Görner et al. 1998; Garreau et al. 2000; Jacquet et al. 2003). This signal transduction pathway under ethanol stress condition was demonstrated (Yamaji et al. 2003; Wang et al. 2004), and accumulation of Msn2p and Msn4p in the nucleus under ethanol stress was

observed (Görner et al. 1998). Intracellular acidification and its interactions with Ira1/2p can also negatively affect the function of Ras proteins to trigger stress responses (Thevelein 1991). It is possible to trigger signal transduction pathway for ethanol tolerance response since ethanol causes intracellular acidification. High ethanol concentrations in culture may cause osmotic stress to yeast cells. Up-regulated expressions of *GPD1*, *HOR2*, *HOR7*, *DAK1*, and *GRE3* were observed under ethanol stress (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu 2010a), which were all dependent on HOG-MAPK signal transduction pathways (Rep et al. 2000). Expression of *MSN2* and *MSN4* can be regulated by HOG-MAPK signal transduction (Hohmann 2002). Thus, HOG-MAPK signal transduction pathway may also be involved in ethanol stress response (Alexandre et al. 2001).

Ethanol has also been shown to induce ROS, and then oxidative stress is possibly imposed on cells indirectly (Du and Takagi 2007). Yap1p is the major oxidative stress regulator (Rodrigues-Pousada et al. 2004, 2010). The N-terminal region of Yap1p contains a NLS, while the C-terminal region contains a nuclear export signal (NES) (Rodrigues-Pousada et al. 2010). In the absence of oxidative stress, Yap1p is exported from the nucleus to cytoplasm via Crm1p (Yan et al. 1998). Under oxidative stress, Yap1p is activated by conformation change through the multistep formation of disulfide bonds via Hyr1p and Ybp1p and transit from the cytoplasm to the nucleus (Rodrigues-Pousada et al. 2010). Transcription factor Hsf1p regulates transcription of many genes in response to heat shock and other stresses (Hahn et al. 2004). For unstressed cells, Hsf1p is constitutively phosphorylated, but under certain stresses, it becomes hyperphosphorylated and adopts an activated conformation to activate transcription of target genes (Lee et al. 2000; Hashikawa et al. 2006). It was suggested that Hsf1p activity might be negatively regulated by cAMP-dependent kinase PKA (Ferguson et al. 2005). Function of Hsf1p in response to ethanol stress was demonstrated by Takemori et al. (2006) that mutant of *HSF1* deletion showed repressed expression for its target genes usually induced by ethanol.

8.2 Transcription Factors and Stress Response Element

Analysis of up-regulated genes by ethanol challenges found many genes share transcription factor binding sites of Msn2p/Msn4p, Yap1p, and Hsf1p in their upstream sequence (Teixeira et al. 2006; Ma and Liu 2010a). Among the 200 up-regulated genes reported under ethanol stress, 58 genes are co-regulated by these three transcription factors, and Msn2p/Msn4p regulates more genes than Yap1p and Hsf1p (Ma and Liu 2010b). Transcription factors Msn2p/Msn4p, Yap1p, and Hsf1p appeared as key regulators for ethanol tolerance response in yeast.

Ethanol stress, as a general stress, activates Msn2p/Msn4p via signal transduction pathways as discussed above to trigger the so-called environmental stress response (Fig. 4). The activated Msn2p/Msn4p induces gene expression via binding

to stress response element (STRE) to trigger stress response (Marchler et al. 1993; Schüller et al. 1994; Martínez-Pastor et al. 1996). STRE has a core pentameric *cis*-acting sequence CCCCT and function in both orientations (Marchler et al. 1993). STRE was found in the upstream sequence of at least 134 genes which displayed enhanced expression under ethanol stress (Ma and Liu 2010b). Although a single copy of STRE elements is sufficient to activate expression of a reporter gene by a stress factor, two or more copies of this sequence can induce a greater expression of stress response genes (Kobayashi and McEntee 1993). However, more copy numbers of STRE elements do not necessarily lead to greater expressions than low copy numbers do, and the presence of a STRE-like element in a promoter region does not imply the functionality of this sequence either. A comprehensive relation should be taken in the light of the STRE position, its copy numbers, and other motifs associated with other transcription factors in the promoter sequence of a target gene.

A double gene deletion *msn2msn4*-mutant showed hypersensitivity to multiple environmental stress conditions, including higher ethanol concentrations (Moskvina et al. 1998). Msn2p was required for the elevated expression of the STRE-controlled genes such as *HSP12* in ethanol-tolerant strain K11, and overexpression of *MSN2* under the control of constitutive promoters such as *TDH3* has shown increased tolerance to ethanol (Watanabe et al. 2007, 2009). However, ethanol-tolerant strains showed slower cell growth, and cell growth is usually affected by either activation of Msn2p or its overexpression under the constitutive promoter control (Hara et al. 1976b; Martínez-Pastor et al. 1996; Durchschlag et al. 2004; Ma and Liu 2010a). This inhibition of cell growth is partly due to negative regulation of *RIM15* via cAMP-PKA signal transduction pathway and *YAK1* (Hartley et al. 1994; Reinders et al. 1998; Lee et al. 2008). *SPI1*, with three STRE sequences in its promoter region, was induced at the stationary phase by Msn2p (Puig and Pérez-Ortín 2000). Induced expression of Msn2p under *SPI1* gene promoter control achieved autoregulated expression of Msn2p, which avoided cell growth inhibition at early stage and showed improved resistance to multiple stresses (including ethanol stress) at stationary phase (Cardona et al. 2007).

Yap1p, a basic leucine zipper transcription factor, is the major oxidative stress regulator required for oxidative stress response. It binds to Yap1p response elements (YRE), including TTASTMA and TTAGTMAGC, and TTACTTA is the preferred binding site (Fernandes et al. 1997; Nguyên et al. 2001; Harbison et al. 2004). At least 105 genes displaying enhanced expression under ethanol stress were found to have YRE elements (Ma and Liu 2010b). Functions of Yap1p in ethanol tolerance are not well documented to date. By forming a homotrimer through the hydrophobic repeat regions, Hsf1p recognizes and binds to conserved heat shock elements (HSE) consisting of inverted 5′-nGAAn-3′ repeats in promoter regions of its target genes (Bonner et al. 1994; Harbison et al. 2004). HSEs are grouped into three categories depending on the organization of the nGAAn motifs. The perfect-type HSE consists of three or more contiguous inverted repeats of the unit (nTTCnnGAAnnTTCn), the gap-type HSE consists of two inverted units separated from a third unit by a 5-bp gap (nTTCnnGAAn(5 bp)nGAAn), and the

step-type HSE consists of direct repeats of the nGAAn or nTTCn motif separated by ((bp)nGAAn(5 bp)nGAAn(5 bp)nGAAn) (Yamamoto et al. 2005).

Regulation of gene expression by transcription factors Msn2p/Msn4p, Yap1p, and Hsf1p is occurred at either transcription level or protein function level via conformation change and location to nucleus as discussed above. Transcription dynamic analyses of *MSN2*, *YAP1*, and *HSF1* under ethanol challenges demonstrated higher expressions of these transcription factors in a short-time period for the parental strain Y-50049 that led to extremely high expression of some regulons such as *HSP26* and *HSP30* as response to ethanol stress (Ma and Liu 2010a). However, it slowed down cell growth, led cells to enter into stationary phase, and didn't build up a culture to finish fermentation (Fig. 5). On the contrary, expression of *MSN2*, *YAP1*, and *HSF1* for ethanol-tolerant strain Y-50316 was moderately repressed or near normally expressed at the early stage and significantly higher at a later stage. This indicates an ethanol tolerance. Gradually increased expression of *MSN4* in ethanol-tolerant strain but repressed in the parental strain suggested a more important role of Msn4p in the dynamic response to ethanol tolerance (Fig. 5). Some induced genes such as *HSP12*, *HSP31*, *HSP32*, *HSP150*,

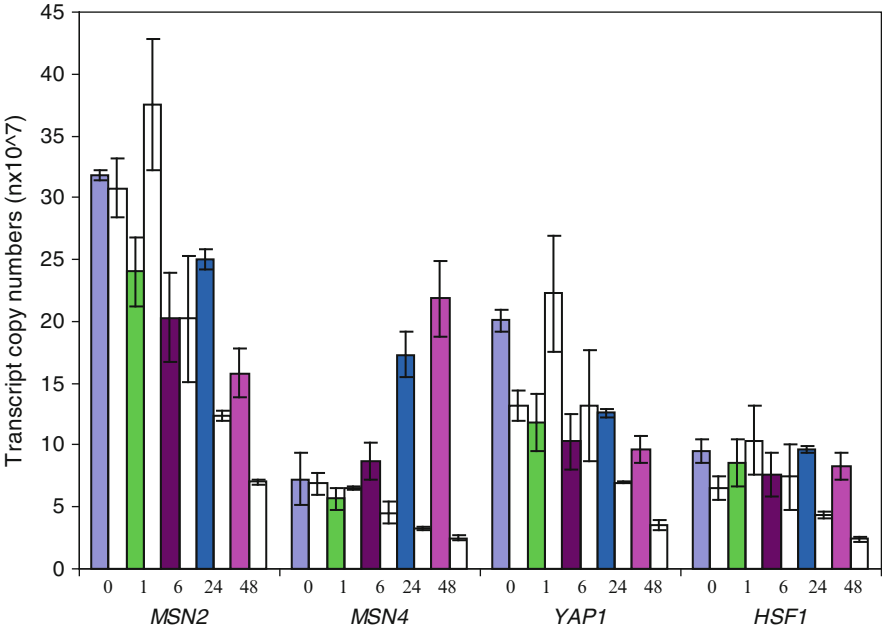

Fig. 5 Comparisons of transcription expression in gene copy numbers ($n \times 10^7$) for selective transcription factor genes between *Saccharomyces cerevisiae* ethanol- and inhibitor-tolerant mutant NRRL Y-50316 and its parental strain NRRL Y-50049 under ethanol challenge over time. Mean values are presented with *error bars* of standard deviations. Values at different time points are presented by a *specific colored bar* as shown in legends for the tolerant strain Y-50316 and an immediately *adjacent open bar* on its right for the parental strain Y-50049 at the same time point

GPH1, *TDH1*, and *GND2* showed significantly higher expression in Y-50316 than in Y-50049, and Msn2p/Msn4p binding motifs were found in the promoter sequence of these genes. Whether the expression difference of these genes is caused by *MSN4* remained unknown and further studies on its regulatory roles for ethanol tolerance are needed.

9 Conclusions and Perspectives

Ethanol tolerance of yeast involves several hundred genes at multiple quantitative trait loci and interplays of complex networks at genome level (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Hu et al. 2007; Ma and Liu 2010a). Many genes induced by ethanol are overlapping with genes involving other environmental factors, such as osmotic, heat shocking, chemical toxicity, and oxidative stress. Mechanisms of ethanol tolerance can only be better understood when a comprehensive view of pathways and network events are considered as functional dynamics. Genetic manipulation of one or a few genes is unable to achieve desirable phenotype for ethanol tolerance. Response to ethanol stress is common, and a transient gene expression response to ethanol challenge does not necessarily imply a functional characteristic of ethanol tolerance in yeast. Yeast tolerance to ethanol can be obtained by evolutionary methods, such as stepwise adaption (Hara et al. 1976a; Cakar et al. 2005; Wei et al. 2007; Dinh et al. 2008; Ma and Liu 2010a), global transcription machinery engineering (gTME) (Alper et al. 2006), and genome shuffling (Shi et al. 2009). A snapshot of expression response for yeast at an earlier stage can be similar. However, a tolerant yeast shows distinct dynamics of gene expression and establishes a viable culture that represents a tolerance phenotype. On the other hand, wild type is unable to survive regardless of significance in earlier response. Thus, results of expression dynamics over time are more informative and should be used for mechanism studies.

Acknowledgments The authors are grateful to Michael A. Cotta and Marsha Ebener for reading the manuscript. This work was supported in part by NIFA National Research Initiative Award 2006-35504-17359. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B (2001) Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Lett 498(1):98–103

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314(5805):1565–1568

Auesukaree C, Damnernsawad A, Kruatrachue M, Pokethitiyook P, Boonchird C, Kaneko Y, Harashima S (2009) Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. J Appl Genet 50(3):301–310

Bai FW, Chen LJ, Zhang Z, Anderson WA, Moo-Young M (2004) Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. J Biotechnol 110(3):287–293

Birch RM, Walker GM (2000) Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. Enzyme Microb Technol 26(9–10):678–687

Blázquez MA, Lagunas R, Gancedo C, Gancedo JM (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinase. FEBS Lett 329(1–2):51–54

Bonner JJ, Ballou C, Fackenthal DL (1994) Interactions between DNA-bound trimers of the yeast heat shock factor. Mol Cell Biol 14(1):501–508

Bruinenberg PM, van Dijken JP, Scheffers WA (1983) A theoretical analysis of NADPH production and consumption in yeasts. J Gen Microbiol 129(4):953–964

Cakar ZP, Seker UO, Tamerler C, Sonderegger M, Sauer U (2005) Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res 5(6–7):569–578

Cardona F, Carrasco P, Pérez-Ortín JE, del Olmo M, Aranda A (2007) A novel approach for the improvement of stress resistance in wine yeasts. Int J Food Microbiol 114(1):83–91

Cartwright CP, Veazey FJ, Rose AH (1987) Effect of ethanol on activity of the plasma-membrane ATPase in, and accumulation of glycine by, *Saccharomyces cerevisiae*. J Gen Microbiol 133(4): 857–865

Casey GP, Ingledew WM (1986) Ethanol tolerance in yeasts. Crit Rev Microbiol 13(3):219–280

Chandler M, Stanley GA, Rogers P, Chambers P (2004) A genomic approach to defining the ethanol stress response in the yeast *Saccharomyces cerevisiae*. Ann Microbiol 54(4):427–454

Chi Z, Arneborg N (1999) Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. J Appl Microbiol 86(6):1047–1052

Choi JY, Stukey J, Hwang SY, Martin CE (1996) Regulatory elements that control transcription activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae OLE1* gene. J Biol Chem 271(7):3581–3589

Colombo S, Ma P, Cauwenberg L, Winderickx J, Crauwels M, Teunissen A, Nauwelaers D, de Winde JH, Gorwa MF, Colavizza D, Thevelein JM (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. EMBO J 17(12):3326–3341

Costa V, Amorim MA, Reis E, Quintanilha A, Moradas-Ferreira P (1997) Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. Microbiology 143(5):1649–1656

Costa V, Moradas-Ferreira P (2001) Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. Mol Aspects Med 22(4–5):217–246

Cronin SR, Rao R, Hampton RY (2002) Cod1p/Spf1p is a P-type ATPase involved in ER function and Ca^{2+} homeostasis. J Cell Biol 157(6):1017–1028

D'Amore T, Stewart GG (1987) Ethanol tolerance of yeast. Enzyme Microb Technol 9 (6):322–330

D'Amore T, Panchal CJ, Russell I, Stewart GG (1990) A study of ethanol tolerance in yeast. Crit Rev Biotechnol 9(4):287–304

Del Castillo Agudo L (1992) Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. Appl Microbiol Biotechnol 37(5):647–651

Ding J, Huang X, Zhang L, Zhao N, Yang D, Zhang K (2009) Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 85(2):253–263

Dinh TN, Nagahisa K, Hirasawa T, Furusawa C, Shimizu H (2008) Adaptation of *Saccharomyces cerevisiae* cells to high ethanol concentration and changes in fatty acid composition of membrane and cell size. PLoS ONE 3(7):e2623

Dinh TN, Nagahisa K, Yoshikawa K, Hirasawa T, Furusawa C, Shimizu H (2009) Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioprocess Biosyst Eng 32(5):681–688

Dombek KM, Ingram LO (1986) Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. Appl Environ Microbiol 52(5):975–981

Du X, Takagi H (2007) *N*-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl Microbiol Biotechnol 75(6):1343–1351

Durchschlag E, Reiter W, Ammerer G, Schüller C (2004) Nuclear localization destabilizes the stress-regulated transcription factor Msn2. J Biol Chem 279(53):55425–55432

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24(4):469–486

Ferguson SB, Anderson ES, Harshaw RB, Thate T, Craig NL, Nelson HC (2005) Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*. Genetics 169(3):1203–1214

Fernandes L, Rodrigues-Pousada C, Struhl K (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. Mol Cell Biol 17(12):6982–6993

Forgac M (1998) Structure, function and regulation of the vacuolar (H^+)-ATPases. FEBS Lett 440(3):258–263

François J, Parrou JL (2001) Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25(1):125–145

Fujita K, Matsuyama A, Kobayashi Y, Iwahashi H (2006) The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols. FEMS Yeast Res 6(5):744–750

Garreau H, Hasan RN, Renault G, Estruch F, Boy-Marcotte E, Jacquet M (2000) Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*. Microbiology 146(9):2113–2120

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11(12):4241–4257

Geymonat M, Wang L, Garreau H, Jacquet M (1998) Ssa1p chaperone interacts with the guanine nucleotide exchange factor of Ras Cdc25p and controls the cAMP pathway in *Saccharomyces cerevisiae*. Mol Microbiol 30(4):855–864

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94(1):73–82

Gong Y, Kakihara Y, Krogan N, Greenblatt J, Emili A, Zhang Z, Houry WA (2009) An atlas of chaperone-protein interactions in *Saccharomyces cerevisiae*: implications to protein folding pathways in the cell. Mol Syst Biol 5:275

Görner W, Durchschlag E, Martinez-Pastor MT, Estruch F, Ammerer G, Hamilton B, Ruis H, Schüller C (1998) Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev 12(4):586–597

Görner W, Durchschlag E, Wolf J, Brown EL, Ammerer G, Ruis H, Schüller C (2002) Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. EMBO J 21(1–2):135–144

Gulshan K, Moye-Rowley WS (2007) Multidrug resistance in fungi. Eukaryot Cell 6(11): 1933–1942

Hahn JS, Hu Z, Thiele DJ, Iyer VR (2004) Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol 24(12):5249–5256

Hamel P, Saint-Georges Y, de Pinto B, Lachacinski N, Altamura N, Dujardin G (2004) Redundancy in the function of mitochondrial phosphate transport in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. Mol Microbiol 51(2):307–317

Hara S, Sasaki M, Obata T, Noshiro K (1976a) Isolation of ethanol tolerant mutants from sake yeast Kyotai no. 7. J Brew Soc Jpn 71:301–304

Hara S, Yamamoto N, Fukuda Y, Obata T, Noshiro K (1976b) Comparison of physiological characteristics between sake yeast Kyokai no. 7 and its ethanol tolerant mutant. J Brew Soc Jpn 71:564–568

Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, Macisaac KD, Danford TW, Hannett NM, Tagne JB, Reynolds DB, Yoo J, Jennings EG, Zeitlinger J, Pokholok DK, Kellis M, Rolfe PA, Takusagawa KT, Lander ES, Gifford DK, Fraenkel E, Young RA (2004) Transcriptional regulatory code of a eukaryotic genome. Nature 431(7004):99–104

Hartley AD, Ward MP, Garrett S (1994) The Yak1 protein kinase of *Saccharomyces cerevisiae* moderates thermotolerance and inhibits growth by an Sch9 protein kinase-independent mechanism. Genetics 136(2):465–474

Hashikawa N, Mizukami Y, Imazu H, Sakurai H (2006) Mutated yeast heat shock transcription factor activates transcription independently of hyperphosphorylation. J Biol Chem 281(7): 3936–3942

Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2007) Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. J Biotechnol 131(1):34–44

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66(2):300–372

Hou J, Lages NF, Oldiges M, Vemuri GN (2009) Metabolic impact of redox cofactor perturbations in *Saccharomyces cerevisiae*. Metab Eng 11(4–5):253–261

Hu XH, Wang MH, Tan T, Li JR, Yang H, Leach L, Zhang RM, Luo ZW (2007) Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae*. Genetics 175 (3):1479–1487

Inoue T, Wang Y, Jefferies K, Qi J, Hinton A, Forgac M (2005) Structure and regulation of the V-ATPases. J Bioenerg Biomembr 37(6):393–398

Jacquet M, Renault G, Lallet S, De Mey J, Goldbeter A (2003) Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*. J Cell Biol 161(3):497–505

Jungwirth H, Kuchler K (2006) Yeast ABC transporters– a tale of sex, stress, drugs and aging. FEBS Lett 580(4):1131–1138

Kaino T, Takagi H (2008) Gene expression profiles and intracellular contents of stress protectants in *Saccharomyces cerevisiae* under ethanol and sorbitol stresses. Appl Microbiol Biotechnol 79(2):273–283

Kitagaki H, Shimoi H (2007) Mitochondrial dynamics of yeast during sake brewing. J Biosci Bioeng 104(3):227–230

Kobayashi N, McEntee K (1993) Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. Mol Cell Biol 13(1):248–256

Kubota S, Takeo I, Kume K, Kanai M, Shitamukai A, Mizunuma M, Miyakawa T, Shimoi H, Iefuji H, Hirata D (2004) Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol. Biosci Biotechnol Biochem 68(4):968–972

Lee S, Carlson T, Christian N, Lea K, Kedzie J, Reilly JP, Bonner JJ (2000) The yeast heat shock transcription factor changes conformation in response to superoxide and temperature. Mol Biol Cell 11(5):1753–1764

Lee P, Cho BR, Joo HS, Hahn JS (2008) Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Mol Microbiol 70(4):882–895

Legras JL, Merdinoglu D, Cornuet JM, Karst F (2007) Bread, beer and wine: diversity reflects human history. Mol Ecol 16(10):2091–2102

Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69(6):627–642

Liu Z, Saha BC, Slininger PJ (2008) Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Wall J, Harwood C, Demain A (eds) Bioenergy. ASM Press, Washington DC, pp 17–36

Lockshon D, Surface LE, Kerr EO, Kaeberlein M, Kennedy BK (2007) The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics 175(1):77–91

López-Ribot JL, Chaffin WL (1996) Members of the Hsp70 family of proteins in the cell wall of *Saccharomyces cerevisiae*. J Bacteriol 178(15):4724–4726

Ma M, Liu ZL (2010a) Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol 10:169

Ma M, Liu ZL (2010b) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 87(3):829–845

Maggio A, Miyazaki S, Veronese P, Fujita T, Ibeas JI, Damsz B, Narasimhan ML, Hasegawa PM, Joly RJ, Bressan RA (2002) Does proline accumulation play an active role in stress-induced growth reduction. Plant J 31(6):699–712

Mansure JJ, Panek AD, Crowe LM, Crowe JH (1994) Trehalose inhibits ethanol effects on intact yeast cells and liposomes. Biochim Biophys Acta 1191(2):309–316

Marchler G, Schüller C, Adam G, Ruis H (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J 12(5):1997–2003

Marks VD, Ho Sui SJ, Erasmus D, van der Merwe GK, Brumm J, Wasserman WW, Bryan J, van Vuuren HJ (2008) Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res 8(1):35–52

Marini AM, Soussi-Boudekou S, Vissers S, Andre B (1997) A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol Cell Biol 17(8):4282–4293

Martin CE, Oh CS, Jiang Y (2007) Regulation of long chain unsaturated fatty acid synthesis in yeast. Biochim Biophys Acta 1771(3):271–285

Martínez-Pastor MT, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J 15(9):2227–2235

McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, Frydman J (2007) Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. Cell 131(1):121–135

Millar DG, Griffiths-Smith K, Algar E, Scopes RK (1982) Activity and stability of glycolytic enzymes in the presence of ethanol. Biotechnol Lett 9(4):601–606

Moskvina E, Schüller C, Maurer CT, Mager WH, Ruis H (1998) A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. Yeast 14(11): 1041–1050

Moukadiri I, Zueco J (2001) Evidence for the attachment of Hsp150/Pir2 to the cell wall of *Saccharomyces cerevisiae* through disulfide bridges. FEMS Yeast Res 1(3):241–245

Müller D, Exler S, Aguilera-Vázquez L, Guerrero-Martín E, Reuss M (2003) Cyclic AMP mediates the cell cycle dynamics of energy metabolism in *Saccharomyces cerevisiae*. Yeast 20(4):351–367

Nabais RC, Sá-Correia I, Viegas CA, Novais JM (1988) Influence of calcium ion on ethanol tolerance of *Saccharomyces bayanus* and alcoholic fermentation by yeasts. Appl Environ Microbiol 54(10):2439–2446

Nguyên DT, Alarco AM, Raymond M (2001) Multiple Yap1p-binding sites mediate induction of the yeast major facilitator *FLR1* gene in response to drugs, oxidants, and alkylating agents. J Biol Chem 276(2):1138–1145

Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I, Brown AJ (2009) Phylogenetic diversity of stress signalling pathways in fungi. BMC Evol Biol 9:44

Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shiomoi H, Ito K (2000) Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J Biosci Bioeng 90(3):313–320

Outlaw J, Collins KJ, Duffield JA (2005) Agriculture as a producer and consumer of energy. CABI Publishing, Oxfordshire/Cambridge

Park H, Bakalinsky AT (2000) SSU1 mediates sulphite efflux in *Saccharomyces cerevisiae*. Yeast 16(10):881–888

Parsell DA, Kowal AS, Singer MA, Lindquist S (1994) Protein disaggregation mediated by heat-shock protein Hsp104. Nature 372(6505):475–478

Pascual C, Alonso A, García I, Romay C (1988) Effect of ethanol on glucose transport, key glycolytic enzymes and proton extrusion in *Saccharomyces cerevisiae*. Biotechnol Bioeng 32(3):374–378

Picard D (2002) Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 59(10):1640–1648

Pina C, António J, Hogg T (2004) Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts by progressive inactivation. Biotechnol Lett 26(19):1521–1527

Piper PW, Talreja K, Panaretou B, Moradas-Ferreira P, Byrne K, Praekelt UM, Meacock P, Récnacq M, Boucherie H (1994) Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. Microbiology 140(11):3031–3038

Piper PW (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol Lett 134(2–3):121–127

Piper PW, Ortiz-Calderon C, Holyoak C, Coote P, Cole M (1997) Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H^+-ATPase. Cell Stress Chaperones 2(1):12–24

Portnoy ME, Schmidt PJ, Rogers RS, Culotta VC (2001) Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. Mol Genet Genomics 265(5):873–882

Prodromou C, Pearl LH (2003) Structure and functional relationships of Hsp90. Curr Cancer Drug Targets 3(5):301–323

Puig S, Pérez-Ortín JE (2000) Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. Yeast 16(2):139–148

Ramírez J, Ramírez O, Saldaña C, Coria R, Peña A (1998) A *Saccharomyces cerevisiae* mutant lacking a K^+/H^+ exchanger. J Bacteriol 180(22):5860–5865

Reinders A, Bürckert N, Boller T, Wiemken A, De Virgilio C (1998) *Saccharomyces cerevisiae* cAMP dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. Genes Dev 12(18):2943–2955

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem 275(12): 8290–8300

Rodrigues-Pousada CA, Nevitt T, Menezes R, Azevedo D, Pereira J, Amaral C (2004) Yeast activator proteins and stress response: an overview. FEBS Lett 567(1):80–85

Rodrigues-Pousada C, Menezes RA, Pimentel C (2010) The Yap family and its role in stress response. Yeast 27(5):245–258

Rosa MF, Sá-Correia I (1996) Intracellular acidification does not account for inhibition of *Saccharomyces cerevisiae* growth in the presence of ethanol. FEMS Microbiol Lett 135(2–3): 271–274

Rudolph AS, Crowe JH (1985) Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiology 22(4):367–377

Sajbidor J, Ciesarová Z, Smogrovicová D (1995) Influence of ethanol on the lipid content and fatty acid composition of *Saccharomyces cerevisiae*. Folia Microbiol 40(5):508–510

Sales K, Brandt W, Rumbak E, Lindsey G (2000) The LEA-like protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. Biochim Biophys Acta 1463(2):267–278

Samuel D, Kumar TKS, Ganesh G, Jayaraman G, Yang PW, Chang MM, Trivedi VD, Wang SL, Hwang KC, Chang DK, Yu C (2000) Proline inhibits aggregation during protein refolding. Protein Sci 9(2):344–352

Sánchez OJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99(13):5270–5295

Schrader M, Fahimi HD (2004) Mammalian peroxisomes and reactive oxygen species. Histochem Cell Biol 122(4):383–393

Scheffler IE (1999) Mitochondria. Wiley-Liss, New York

Schüller C, Brewster JL, Alexander MR, Gustin MC, Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. EMBO J 13(18):4382–4389

Sebollela A, Louzada PR, Sola-Penna M, Sarone-Williams V, Coelho-Sampaio T, Ferreira ST (2004) Inhibition of yeast glutathione reductase by trehalose: possible implications in yeast survival and recovery from stress. Int J Biochem Cell Biol 36(5):900–908

Shi DJ, Wang CL, Wang KM (2009) Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 36(1):139–147

Singer MA, Lindquist S (1998) Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. Mol Cell 1(5):639–648

Stukey JE, McDonough VM, Martin CE (1989) Isolation and characterization of *OLE1*, a gene affecting fatty acid desaturation from *Saccharomyces cerevisiae*. J Biol Chem 264(28): 16537–16544

Stukey JE, McDonough VM, Martin CE (1990) The *OLE1* gene of *Saccharomyces cerevisiae* encodes the $\triangle 9$ fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem 265(33):20144–20149

Takagi H, Takaoka M, Kawaguchi A, Kubo Y (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71(12):8656–8662

Takagi H, Matsui F, Kawaguchi A, Wu H, Shimoi H, Kubo Y (2007) Construction and analysis of self-cloning sake yeasts that accumulate proline. J Biosci Bioeng 103(4):377–380

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations and biotechnological applications. Appl Microbiol Biotechnol 81(2):211–223

Takemori Y, Sakaguchi A, Matsuda S, Mizukami Y, Sakurai H (2006) Stress-induced transcription of the endoplasmic reticulum oxidoreductin gene *ERO1* in the yeast *Saccharomyces cerevisiae*. Mol Genet Genomics 275(1):89–96

Teixeira MC, Monteiro P, Jain P, Tenreiro S, Fernandes AR, Mira NP, Alenquer M, Freitas AT, Oliveira AL, Sá-Correia I (2006) The YEASTRACT database: a tool for the analysis of transcription regulatory associations in *Saccharomyces cerevisiae*. Nucleic Acids Res 34: D446–D451

Teixeira MC, Raposo LR, Mira NP, Lourenço AB, Sá-Correia I (2009) Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. Appl Environ Microbiol 75(18):5761–5772

Thevelein JM (1991) Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signaling pathway in yeast: the relationship to nutrient-induced cell cycle control. Mol Microbiol 5(6):1301–1307

Thevelein JM, de Winde JH (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 33(5):904–918

Thevelein JM, Cauwenberg L, Colombo S, De Winde JH, Donation M, Dumortier F, Kraakman L, Lemaire K, Ma P, Nauwelaers D, Rolland F, Teunissen A, Van Dijck P, Versele M, Wera S, Winderickx J (2000) Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. Enzyme Microb Technol 26(9–10):819–825

Van Voorst F, Houghton-Larsen J, Jønson L, Kielland-Brandt MC, Brandt A (2006) Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress. Yeast 23(5):351–359

Vertes A, Qureshi N, Yukawa H, Blaschek H (2009) Biomass to biofuels. John Wiley & Sons, Ltd, West Sussex

Walker GM, Maynard AI (1997) Accumulation of magnesium ions during fermentative metabolism in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 18(1):1–3

Wang L, Renault G, Garreau H, Jacquet M (2004) Stress induces depletion of Cdc25p and decreases the cAMP producing capability in *Saccharomyces cerevisiae*. Microbiology 150(10):3383–3391

Watanabe M, Tamura K, Magbanua JP, Takano K, Kitamoto K, Kitagaki H, Akao T, Shimoi H (2007) Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol-tolerance sake yeast Kyokai no. 11. J Biosci Bioeng 104(3):163–170

Watanabe M, Watanabe D, Akao T, Shimoi H (2009) Overexpression of *MSN2* in a sake yeast strain promotes ethanol tolerance and increases ethanol production in sake brewing. J Biosci Bioeng 107(5):516–518

Wei P, Li Z, Lin Y, He P, Jiang N (2007) Improvement of the multiple-stress tolerance of an ethanologenic *Saccharomyces cerevisiae* strain by freeze-thaw treatment. Biotechnol Lett 29(10):1501–1508

Wilson WA, St Amour CV, Collins JL, Ringe D, Petsko GA (2004) The 1.8-A resolution crystal structure of YDR533Cp from *Saccharomyces cerevisiae*: a member of the DJ-1/ThiJ/PfpI superfamily. Proc Natl Acad Sci USA 101(6):1531–1536

Xiao D, Wu S, Zhu X, Chen Y, Guo X (2010) Effects of soya fatty acids on cassava ethanol fermentation. Appl Biochem Biotechnol 160(2):410–420

Yamaji K, Hara S, Mizoguchi H (2003) Influence of Ras function on ethanol stress response of sake yeast. J Biosci Bioeng 96(5):474–480

Yamamoto A, Mizukami Y, Sakurai H (2005) Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in *Saccharomyces cerevisiae*. J Biol Chem 280(12):11911–11919

Yan C, Lee LH, Davis LI (1998) Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. EMBO J 17(24):7416–7429

Yoshikawa K, Tanaka T, Furusawa C, Nagahisa K, Hirasawa T, Shimizu H (2009) Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in *Saccharomyces cerevisiae*. FEMS Yeast Res 9(1):32–44

You KM, Rosenfield CL, Knipple DC (2003) Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl Environ Microbiol 69(3): 1499–1503

Young JC, Agashe VR, Siegers K, Hartl FU (2004) Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol 5(10):781–791

Yuan DS, Dancis A, Klausner RD (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. J Biol Chem 272(41):25787–25793

Zhao XQ, Xue C, Ge XM, Yuan WJ, Wang JY, Bai FW (2009) Impact of zinc supplementation on the improvement of ethanol tolerance and yield of self-flocculating yeast in continuous ethanol fermentation. J Biotechnol 139(1):55–60

High Gravity Ethanol Fermentations and Yeast Tolerance

F.W. Bai and X.Q. Zhao

Contents

1 Introduction 118
2 Stresses and Their Impact on Yeast Cells 119
3 Ethanol Production from Sugar- and Starch-Based Feedstocks 120
3.1 Osmotic Stress 120
3.2 Ethanol Stress 123
3.3 Thermal Stress 124
3.4 Industrial Processing Stresses 125
4 Evolutionary Engineering 126
5 Bioprocess Engineering 126
5.1 Medium Optimization 127
5.2 High Cell Density and Immobilized Cells 128
5.3 Consecutive Batch Fermentation 129
5.4 Process Oscillation 130
6 Conclusions and Perspectives 131
References 132

Abstract High gravity (HG) fermentations save energy consumption for both ethanol distillation and subsequent discharge treatment. However, yeast cells suffer from various stresses under HG conditions, which often result in stuck or sluggish fermentations with more sugars remained unfermented, and thus reduce efficiency of ethanol fermentation. This chapter focuses on stresses affecting ethanol fermentations under HG conditions and their impact on yeast growth and ethanol production. The HG condition associated with osmotic pressure may repress yeast cells for ethanol fermentation from sugar-based feedstocks such as molasses but less likely for starch-based feedstocks that are fermented by simultaneous saccharification and fermentation process in industry. However, ethanol inhibition is a major stress for

F.W. Bai (✉) • X.Q. Zhao
Department of Bioscience and Bioengineering, Dalian University of Technology, Dalian, China
e-mail: fwbai@dlut.edu.cn

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_5,

ethanol fermentation from both sugar- and starch-based feedstocks in which ethanol concentration as high as 12–14% (v/v) can be commonly achieved, and higher ethanol concentration of more than 15% (v/v) is expected. On the other hand, it is a less concern for ethanol fermentation from hydrolysate of lignocellulosic biomass since ethanol concentration achieved is usually lower than 12% (v/v), a tolerable level for ethanologenic yeast. Instead, overcoming the inhibition of toxic by-products generated during biomass pretreatment is a major issue for ethanol production from lignocellulosic biomass. Strategies for developing stress-tolerant strains and bioprocess engineering aspects to alleviate the impact of stresses on yeast cells are discussed.

1 Introduction

High gravity (HG) fermentations save energy consumption for ethanol distillation and subsequent distillage treatment, particularly when the distillage is treated by energy intensive multi-evaporation process for ethanol production from grain-based feedstocks (Bai et al. 2004). The HG research and development have been attracted interest to both academia and industry, although criteria to define HG fermentations are ambiguous. In general, for ethanol production from starch-based feedstocks, HG mash containing 25–30% (w/v) solids has been widely used in industry to achieve ethanol concentration as high as 12–14% (v/v). On the other hand, very high gravity (VHG) mash with solids in excess of 35% (w/v) has been explored by academia to achieve more than 15% (v/v) ethanol (Thomas et al. 1995; Devantier et al. 2005a, b). As for ethanol fermentation from sugar-based feedstocks such as molasses, HG or VHG fermentation can be carried out in terms of equivalent sugar concentration in the medium and ethanol achieved within the fermentation system. However, ethanol production from lignocellulosic biomass is different, and ethanol concentration is less likely higher than 12% (v/v) since the viscous slurry may cause problems in pipeline transportation as well as heat and mass transfer.

Due to their unique characteristics, strains of *Saccharomyces cerevisiae* have dominated ethanol fermentations from sugar- and starch-based feedstocks for a long history, which are is still the primary consideration for ethanol production from lignocellulosic biomass with proper genetic modifications to expand their substrate spectrum to utilize C-5 sugars liberated from the hydrolysis of hemicellulosic component (van Vleet and Jeffries 2009), although other species like *Zymomonas mobilis* are also under development. See Chap. 6 for more detailed discussions on improving biomass sugar utilization by engineered *S. cerevisiae*. Under HG or VHG fermentation conditions, yeast cells suffer from various stresses including ethanol inhibition when sugar- and starch-based feedstocks are used, which detrimentally affect cell growth, viability and fermentability, and even lead to stuck or sluggish fermentations with more sugars remained unconverted (Ingledew 2003). This chapter addresses stresses associated with ethanol

fermentations by *S. cerevisiae* under HG and VHG conditions, as well as strategies for developing stress-tolerant strains and bioprocess engineering aspects to alleviate the stress impact.

2 Stresses and Their Impact on Yeast Cells

Yeast stresses during ethanol fermentation can be categorized as follows: (1) harmful by-products or inhibitors generated in the pretreatment of feedstock, particularly for lignocellulosic biomass; (2) high ethanol concentrations achieved during ethanol fermentations from sugar- and starch-based feedstocks; (3) osmotic effect exerted by sugar-based feedstocks; and (4) process parameters such as temperature and pH value that are significantly deviated from physiological optimums of yeast cells.

The impact of these stresses on yeast cell growth and ethanol fermentation is significant and complex, which affects process design and economics to a large extent. For example, the severity of the pretreatment of lignocellulosic biomass and whether detoxification is needed for the hydrolysate are dependent on the by-products produced during the process and their inhibitory effect on yeast cells. The cascade system designed for continuous ethanol fermentation in industry is mainly for alleviating ethanol inhibition since ethanol concentration increases onward and the highest ethanol concentration is achieved only within the last tank. Therefore, identification of stresses associated with different fermentation systems and understanding of their impact on yeast cells are prerequisites for developing strategies to improve stress tolerance for more efficient ethanol production.

Ethanol production at large scale from starch-based feedstocks is not sustainable since it drives up the market prices of grains and related food products that threats food security worldwide (Rosamond et al. 2007). Recently, intensive studies have been focused on ethanol production from agricultural wastes, particularly lignocellulosic biomass that is abundantly available with less impact on grain production and food supply (Service 2007). Unlike starch-based feedstocks, ethanol production from lignocellulosic biomass is not suitable to be carried out under VHG conditions. It is mainly because the tricky component lignin makes the slurry extremely viscous that may cause numerous engineering problems in pipeline transportation as well as heat and mass transfer. Thus, HG fermentation with ethanol concentration no more than 12% (v/v) is technically and economically preferred. Therefore, ethanol inhibition is not a main stress for *S. cerevisiae* since it is tolerable to ethanol at that level. However, to liberate sugars from this kind of biomass for ethanol fermentation, a significant technical challenge of lignocellulose degradation has to be addressed (Himmel et al. 2007). A pretreatment under harsh conditions is commonly required, which inevitably generates numerous inhibitory by-products that are toxic to yeast cells. Categories of the toxic by-products and their concentrations in the hydrolysate are depending upon the characteristics of feedstocks as well as pretreatment methods applied.

Comprehensive reviews on the inhibitory compounds, microbial response, and inhibitor detoxification are available elsewhere (Pienkos and Zhang 2009; Liu and Blaschek 2010; Parawira and Tekere 2011). See Chap. 1 for more discussions on molecular mechanisms of in situ detoxification by yeast.

3 Ethanol Production from Sugar- and Starch-Based Feedstocks

Although VHG media containing total solids as high as 350 g/L are needed in order to achieve more than 15% (v/v) ethanol at the end of the fermentation, osmotic stress from sugars is less likely to occur on yeast cells for ethanol production from starch-based feedstocks. During the widely used simultaneous saccharification and fermentation (SSF) process, sugars released by glucoamylases under ethanol fermentation conditions are consumed and converted into ethanol and CO_2 immediately by yeast cells without significant accumulation within the fermentation system. As for ethanol production from sugar-based feedstocks such as molasses, high sugar concentration may exert osmotic effect on yeast cells and affect their ethanol fermentation performance. However, this kind of substrate inhibition can be overcome by developing corresponding process engineering strategies such as a fed-batch mode or continuous fermentation with tanks-in-series systems.

Under fed-batch conditions, ethanol fermentation is initiated by inoculating yeast cells into diluted medium containing sugars of 100–120 g/L. When sugar concentration decreases below a threshold, the VHG medium is fed, keeping sugar concentration with the tank at a relatively low level to prevent substrate inhibition in yeast cells, but ethanol concentration increases continuously until the end of the fermentation. As for continuous fermentation, the tanks-in-series system is commonly used. The VHG medium is fed into the first one or two tanks and diluted immediately so that substrate inhibition in yeast cells can be effectively prevented (Fig. 1). In this example, ethanol concentration increases as the fermentation broth flows through the system, and the highest ethanol concentration is achieved with the mature broth discharged from the last tank. This design alleviates both substrate inhibition and ethanol toxicity in yeast cells at a certain level. However, capital investment on this kind of facilities is significantly higher compared with the batch and fed-batch fermentation systems. It is applied mainly in plants for large scale fuel ethanol production.

3.1 Osmotic Stress

High sugar concentrations in molasses and other sugar-based feedstocks may exert osmotic effect on yeast cells, particularly under VHG conditions. The osmotic stress causes water outflow from yeast cells affecting their growth, possibly by blocking the cell cycle at G1 or G2/M by the downregulation of the kinase

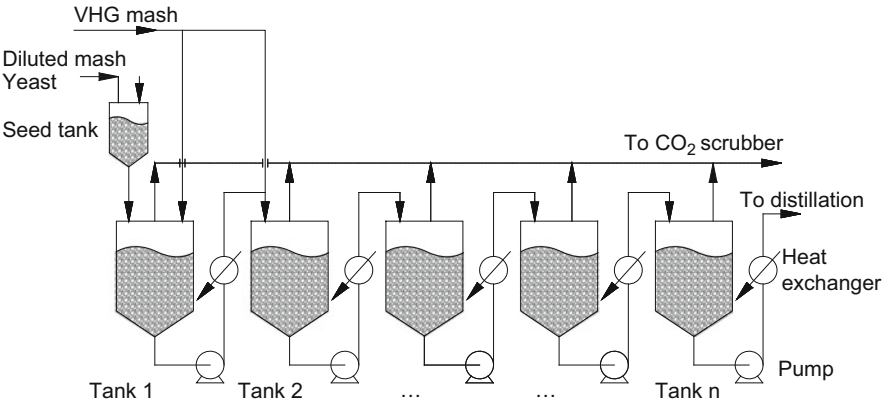

Fig. 1 Continuous VHG ethanol fermentation with a tanks-in-series system to alleviate substrate and ethanol inhibition

Gln3p–Cdc28p activity (Belli et al. 2001) or inhibiting the kinase Clb2p–Cdc28p (Alexander et al. 2001). As a result, ethanol production is inhibited immediately once yeast growth is arrested by osmotic stress. When yeast is exposed to osmotic stress conditions, the compatible solute glycerol is accumulated within cytoplasm to counteract the dehydration effect, which was supported by the osmotic sensitivity of the mutants deficient in the key enzymes of the glycerol biosynthetic pathway (Siderius et al. 2000), especially under anaerobic conditions for ethanol production (Modig et al. 2007). It seems that yeast is able to regulate concentrations of intracellular osmolytes by either metabolic activities to synthesize or degrade them on time or by activating membrane transporters to control their traffic across the membranes (Kayingo et al. 2001).

Knowledge of mechanisms underlying yeast response to osmotic stress is limited. It is known that the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway is triggered by osmotic stress (O'Rourke et al. 2002). In this pathway, there are two sensing branches that activate the MAP kinase (MAPK) cascade module with different mechanisms (Fig. 2). The Sho1 branch needs Sho1 and the mucin-like proteins Hkr1 and Msb2 to detect osmotic stress (Tatebayashi et al. 2007), followed by the involvement of the small G-protein Cdc42 and the p21-activated kinases (PAKs) Ste20 and Cla4. The target of Ste20 is the MAPKKK Ste11, which activates the MAPKK Pbs2 under osmotic stress conditions, resulting in activation of Hog1 (Hohmann 2009). Another branch to the activation of Pbs2 involves a two-component phosphor-relay signaling system with the participation of the transmembrane protein Sln1 and the response regulator proteins Ypd1 and Ssk1, through which two redundant MAPKKKs (Ssk2 and Ssk22) participate in the phosphorylation of Pbs2 for the final activation of Hog1 (Posas et al. 1996; Posas and Saito 1998). Once Hog1 is activated, it coordinates the transcriptional response for yeast cells to adapt to osmotic stress (O'Rourke and Herskowitz 2004).

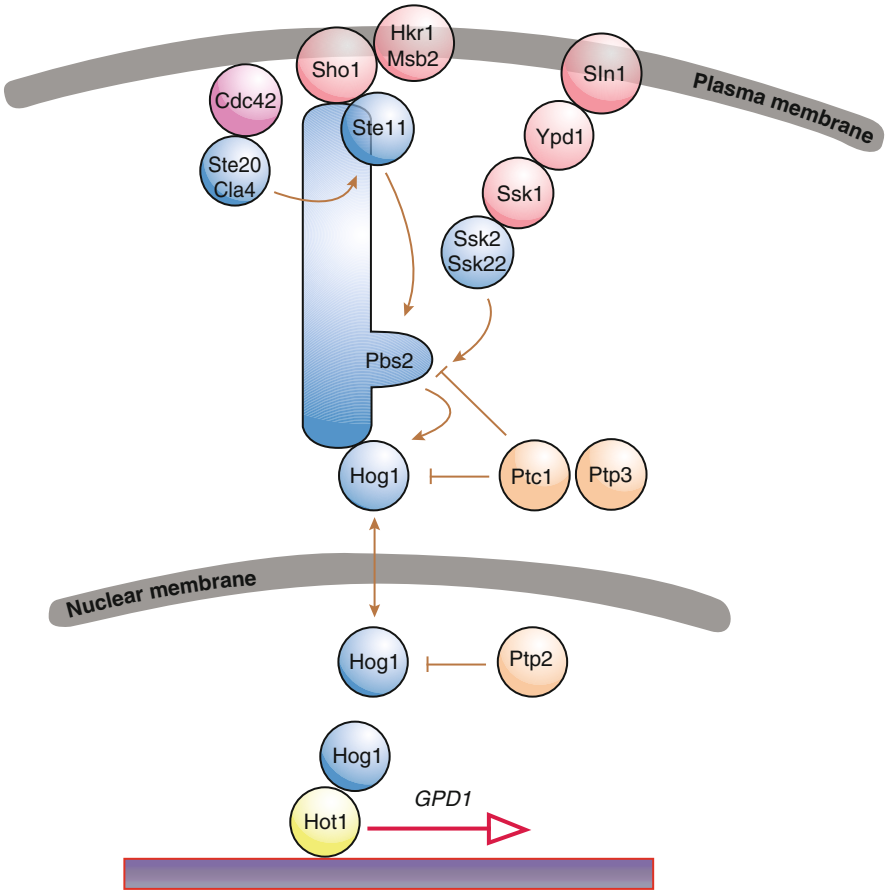

Fig. 2 The yeast HOG pathway. Membrane-localized sensors and regulators are shown in *red*, protein kinases in *blue*, protein phosphatases in *orange*, and transcription factors in *yellow*. Two branches converge at the level of Pbs2 to activate Hog1, which accumulates in the nucleus under stress. Ste11, Ssk2, and Ssk22 are MAPKKKs; Pbs2 is a MAPKK, and Hog1 is the MAPK in the system (This figure is reprinted from Hohmann (2009), with permission from Elsevier)

The consequence of this induction results in the accumulation of glycerol by closing the glycerol export channel Fps1 to decrease extracellular excretion of glycerol (Tamás et al. 1999). It also induces the transcription of genes for glycerol biosynthesis through two paths: (1) the expression of genes *GPD1, GPP1*, and *GPP2* encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively; and (2) the activation of the enzyme phosphofructo-2-kinase, which produces the glycolytic activator fructose-2,6-bisphosphate, and thus increase the rate of glycerol production at the upstream of the glycolytic pathway. In addition, the uptake of glycerol from the surrounding can also be enhanced by the expression of gene *STL1* encoding the glycerol H^+ symporter, a member of the sugar transporter family (Ferreira et al. 2005; Ferreira and Lucas 2007). An overview of the

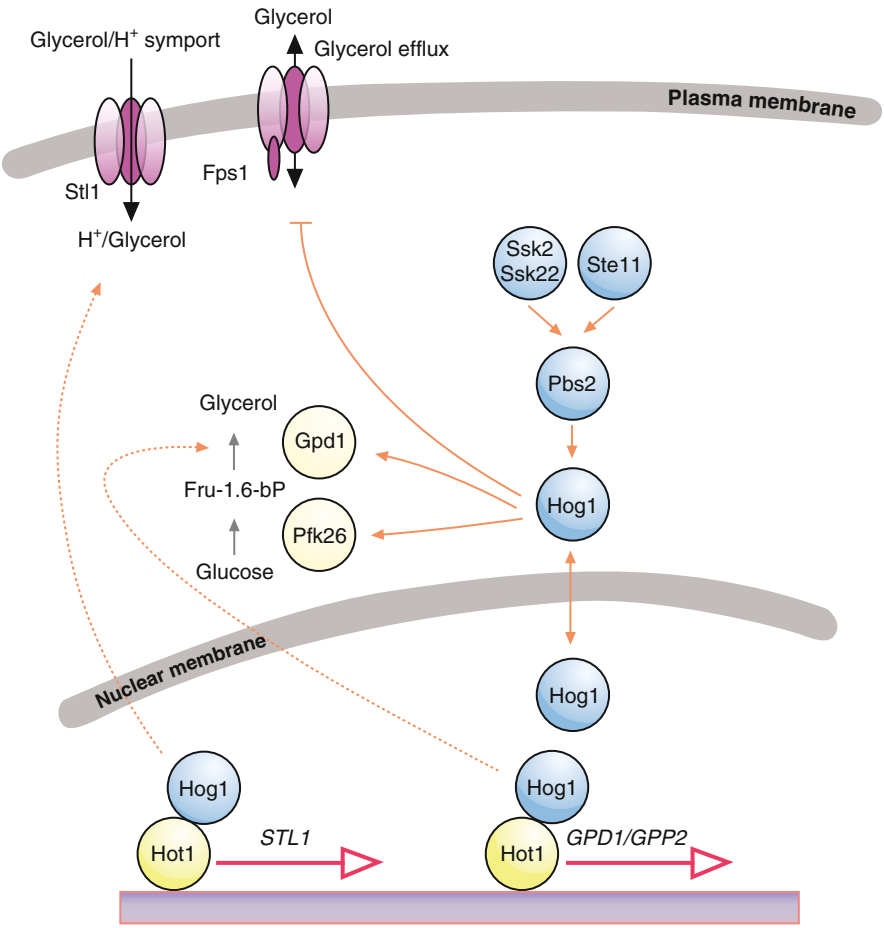

Fig. 3 Mechanisms by which Hog1 controls glycerol accumulation. The HOG pathway is only represented schematically. Protein kinases are indicated in *blue*, transcription factors in *yellow*, enzymes in *green*, and transmembrane transporters in *violet*. *Broken lines* represent protein production rather than regulation (This figure is reprinted from Hohmann (2009), with permission from Elsevier)

process involved in intracellular glycerol accumulation to counteract osmotic stress in yeast is presented (Fig. 3).

3.2 *Ethanol Stress*

Although substrate inhibition in yeast cells can be reduced by process engineering strategies such as fed-batch operation and continuous fermentation with the tanks-in-series system, ethanol inhibition is inevitable, especially near the end of the fed-batch

fermentation or within the rear tanks of the tanks-in-series fermentation system. Such an ethanol inhibition causes stuck or sluggish fermentations with more sugars remained unfermented, which significantly compromise ethanol yield.

The toxic effect of ethanol on cell growth, viability, and fermentability has been observed and studied since the very beginning of the brewery industry. The mechanistic understanding of this phenomenon has experienced different stages with the progress of bioprocess engineering and biological sciences. In the earlier years, ethanol inhibition was quantitatively characterized by incorporating its impact into the kinetics of yeast growth and ethanol production (Aiba et al. 1968). Later, qualitative explanation of this phenomenon was mainly at cellular levels, particularly on its damage to various membrane structures of yeast cells (Casey and Ingledew 1986). Recently, studies of ethanol tolerance have been focused at molecular levels and identification of tolerant candidate genes. For example, decreasing trehalose degradation by the antisense RNA-mediated inhibition of the acid trehalase gene *ATH1* transcription improved ethanol tolerance and fermentability of *S. cerevisiae* (Jung and Park 2005). Overexpression of tryptophan biosynthesis gene *TRP1-5* and tryptophan permease gene *TAT2* also resulted in improved ethanol tolerance (Hirasawa et al. 2007). Similar results were observed by disruption of the cytidine 5′-triphosphate (CTP) synthase gene *URA7* involved in de novo biosynthesis of pyrimidines and the cysteine aminopeptidase gene *GAL6* (Yazawa et al. 2007). Overexpression of *FPS1* encoding the plasma membrane aquaglyceroporin reduced the intracellular accumulation of ethanol and enabled yeast cells to achieve higher ethanol titer with a VHG medium containing 300 g L^{-1} glucose, which represented a 15% increase in ethanol concentration comparing with the wild type strain (Teixeira et al. 2009). A significant amount of efforts has been made in investigation of gene expression and regulatory networks at the genome level (Yoshikawa et al. 2009; Stanley et al. 2010; Ma and Liu 2010). See Chap. 4 for more detailed discussions on molecular mechanisms of ethanol tolerant in yeast.

3.3 Thermal Stress

Fermentation systems operated at temperatures of 35–40°C are preferred in industry since they can be cooled down by cooling water from a regular cooling tower with temperatures about 2–3°C lower than that of the environment, which are usually higher than 35°C in summer for most tropical and subtropical regions where feedstocks for ethanol production are abundant. If chilled water is required, more capital investments on the facilities such as the lithium bromide absorption system are needed in addition to the costs of energy consumption and operation maintenance (Bai et al. 2008).

The optimum temperature for efficient yeast cell growth and ethanol fermentation is ranged from 30°C to 32°C, although some strains are able to tolerate temperatures above 35°C. Under higher temperature conditions, yeast performance on ethanol fermentation is negatively affected or deteriorated due to the synergistic

inhibition of ethanol and high temperature. For a fed-batch fermentation under aeration and good nutritional conditions supplemented with biotin and vitamins, *S. cerevisiae* CBS 8066 was observed to have reduced viability by the synergistic effect of ethanol concentration and fermentation temperature (Aldiguier et al. 2004). With 90% viable cells as the criterion, a threshold of ethanol concentration was detected at 80, 100, and 50 g/L for temperature at 27°C, 30–33°C, and 36°C, respectively. An ethanol concentration of 120 g/L was achieved at 30–33°C by this strain, but it was decreased drastically to 93 g/L at 36°C (Aldiguier et al. 2004).

Biosynthesis of a set of proteins known as the heat shock proteins (HSPs) is rapidly induced when yeast cells are suffered from thermal stress. For example, HSP104 is greatly expressed, which is a molecular chaperone that is not essential for yeast growth at normal temperature but promotes cell survival by disassembling aggregated proteins (Lindquist and Kim 1996; Schirmer et al. 2004; Bösl et al. 2006). Temperature-induced transcription of the HSP genes in *S. cerevisiae* is governed by the transcription factors, which bind to the promoter regions of the HSP genes and influence a broad range of biological functions associated with heat stress such as protein folding and maturation, energy generation, carbohydrate metabolism, integrity maintenance, and cell signaling (Yamamoto et al. 2008).

Mechanisms of yeast thermal tolerance are not clear, and thermal tolerant ethanol-producing strains have not yet become available. Recent applications of high-throughput screening tools such as DNA microarrays revealed significant amount of information on global gene expression under the stress for *S. cerevisiae* (Mensonides et al. 2002; Postmus et al. 2008; Auesukaree et al. 2009). However, most data were generated under laboratory conditions. In practice, the impact of sustained high temperature on yeast cells under industrial conditions is significantly different from that induced by transient heat shocks exerted on yeast cells under laboratory conditions. Therefore, it is necessary to adjust and develop strategies to explore yeast thermal tolerance using fed-batch fermentation or chemostat systems mimic industrial conditions.

3.4 Industrial Processing Stresses

Industrial processing procedures also generate numerous stresses on yeast cells, for example, nutritional depletion. Under industrial conditions, no yeast extract or peptone is used to nourish yeast cells, and almost all sugars are consumed at the end of the fermentation for a high ethanol yield, which is calculated based on the starch or sugars fed into the fermentation system without deduction of the residual sugars (Bai et al. 2008). Low levels of nutrition supplies and quick cell growth may present yeast cells a threat of nutritional depletion.

Unlike the production of value-added fine chemicals and pharmaceuticals such as amino acids and antibiotics, ethanol fermentation is carried out under semi-sterile conditions provided by vigorous propagation of yeast cells at acidic

conditions with a pH value around 4.5, since the energy consumption is economically not acceptable if those huge tanks with working volumes of hundreds, even thousands of cubic meters are sterilized by vapor and operated under sterilized conditions. Thus, contamination of bacteria such as *Acetobacter* and *Lactobacilli* that produce acetic and lactic acids and decrease of the pH value of the fermentation system are inevitable (Narendranath 2003). In addition, the preferred anaerobic ethanol fermentation condition and CO_2 produced and dissolved in the fermentation broth significantly compromise the tolerance of yeast cells to environmental stresses (Arcay-Ledezma and Slaughter 1984).

4 Evolutionary Engineering

Evolutionary engineering has been widely used for tolerant strain development. This approach applies selection pressure on yeast under laboratory conditions mimic natural evolution process to obtain adapted populations or spontaneous mutations. It has been demonstrated to be efficient in the improvement of multiple stress tolerance with yeast. For example, after nine rounds of batch selection for freezing–thawing stress resistance, a multiple stress-tolerant mutant was obtained, which exhibited 62-fold increase in ethanol tolerance, 89-fold increase in thermal resistance, and 1,429-fold increase in oxidative stress tolerance that were characterized by survival percentages of yeast cells (Cakar et al. 2005). Another example is the acquisition of anaerobic xylose utilization ability for the recombinant yeast engineered with heterogeneous xylose metabolic pathway. The recombinant yeast utilized xylose under aerobic conditions only, which is not suitable for ethanol production operated with trace oxygen or anaerobic conditions. Using the evolutionary engineering method through selections over 266 days, about 460 generations in a chemostat system operated from aerobic to microaerobic until finally anaerobic conditions for the recombinant to acquire resistance to oxygen depletion (Sonderegger and Sauer 2003). Technically, strains with specific properties developed by rational approaches such as the recombinant engineered with the xylose metabolic pathway can be subject of evolutionary engineering for further improvement. Strains selected by the evolutionary engineering strategy can also be optimized by rational design (Petri and Schmidt-Dannert 2004).

5 Bioprocess Engineering

With suitable yeast strains in hand, whether naturally selected or genetically modified, bioprocess engineering strategies need to be established to ultimately explore their potentials for more efficient ethanol production. Conventional modes such as batch, fed-batch, continuous systems with single tank or tanks-in-series have been used by industry. These procedures create different environmental

conditions for yeast cells to balance their physiological requirement, fermentation performance, and economic aspects associated with capital investment and energy consumption. Continued efforts on improvement are needed, and novel processes are being developed.

5.1 Medium Optimization

Stuck or sluggish fermentation occurs when sugar utilization rate becomes very slow or fermentation time is protracted significantly, especially toward the end of ethanol fermentation in which high ethanol concentration is achieved, and nutrition is depleted. Nitrogen source, as a macronutrient, is crucial for yeast cells to synthesize proteins and other nitrogenous components. In industry, the nitrogen source is provided naturally with feedstocks, for example, corn is rich in proteins without additional cost. However, for molasses, cassava chips, and hydrolysate of lignocellulosic biomass that are deficient in proteins, ammonia or ammonium sulfate/phosphate or urea that is assimilable to yeast cells should be supplemented. The amount of the supplementation of these nitrogen sources can be estimated by the amount of yeast biomass accumulated during ethanol fermentation. Yeast cells are able to use ammonium as a sole nitrogen source to synthesize all kinds of amino acids and proteins that are required for intracellular metabolism (Magasanik and Kaiser 2002). If ammonium salts or urea that can be broken down by yeast cells into ammonia and water are supplemented properly, the deficiency of amino acids is unlikely to occur under the industry processing conditions.

Recent study demonstrated that supplementation of amino acids significantly enhanced stress tolerance of yeast cells and their growth and thereafter, the performance of ethanol fermentation under VHG conditions (Pham and Wright 2008a, b). Proline has been shown as an effective protectant to multiple stresses including osmotic pressure, ethanol inhibition, and oxidative damage (Takagi et al. 2005; Takagi 2008). However, unlike other amino acids that can be utilized easily, yeast cells are limited in uptaking of proline effectively during ethanol fermentation. Although proline is relatively rich in mashes, especially for hydrolysate supplemented with industrial proteases, proline-specific permease PUT4 was found to be repressed by assimilable nitrogen, particularly by ammonium (Poole et al. 2009). Therefore, the amount of ammonium supplementation needs to be optimized to provide enough assimilable nitrogen at the early and middle stages of ethanol fermentation for yeast cells to propagate quickly but gradually depleted toward the end of the fermentation to prevent ammonium inhibition in proline uptake. Thus, with the increased concentration of ethanol during the fermentation, yeast cells become more tolerant to ethanol inhibition since efficient uptake of proline in the mash protect them effectively.

Yeast cells also need many other micronutrients such as vitamins and inorganic ions which are important regulators and cofactors of numerous enzymes that catalyze their intracellular metabolism. But compared with assimilable nitrogen,

trace levels of these components are adequate to nourish yeast cells, which can be supplied easily with the feedstocks in industry. Since yeast general stress response is mediated to a large extent by global transcription factors Msn2/Msn4, zinc starvation could weaken their response to environmental stresses (Gauci et al. 2009). On the other hand, zinc supplementation exhibited a significant impact on metabolic flux distribution of the self-flocculating yeast SPSC01 during ethanol fermentation under VHG conditions (Table 1), directing more carbon flux to the biosynthesis of ergosterol and trehalose and reducing the production of glycerol, one of the major by-products of ethanol fermentation, thus improved yeast tolerance and ethanol yield (Zhao et al. 2009; Xue et al. 2010).

Table 1 Impact of zinc supplementation on continuous VHG ethanol fermentation with *S. cerevisiae*

Zinc sulfate g/L	Biomass g (DCW)/L	Glucose g/L	Ethanol g/L	Yield	Glycerol g/L	Total ergosterol mg/g(DCW)	Trehalose mg/g(DCW)
0	22.0	0.73	104.1	0.425	5.53	7.86	127.5
0.01	22.0	0.75	107.0	0.437	3.44	12.43	235.5
0.05	22.0	0.63	114.5	0.467	3.21	12.89	255.4
0.10	22.0	0.65	110.8	0.452	3.42	14.76	238.3

The continuous VHG ethanol fermentation was carried out at the dilution rate of 0.025 h^{-1}, and the size of yeast flocs was detected online by the focused beam reflectance measurement (FBRM) system

5.2 High Cell Density and Immobilized Cells

High cell density facilitates biological detoxification of inhibitors such as toxic by-products generated in the pretreatment of lignocellulosic biomass and lactic acids produced by contaminated bacteria, and thus alleviate their stressful impact. High levels of cell density can be achieved by increasing inoculum size to stimulate propagation, recycling cells separated by centrifuges, and yeast cell immobilization. Since yeast seed needs to be prepared with supplementation of various nutritional components under aerobic conditions that consume much more energy, the application of the large inoculum in industry is limited from economic point of view. As for the separation of yeast cells by centrifuges, special processes to remove non-fermentable solid residues in feedstocks to prepare a clear substrate are required, which is not suitable for ethanol production due to the significant loss of sugars with the removal of the residues. It also increases contamination risk associated with these process operations. In addition, a significant capital investment is required for centrifuges and energy consumption for the operation.

High cell density can be obtained when cells are immobilized. Ethanol fermentation with immobilized yeast cells has been intensively studied since the 1970s. Unfortunately, no commercial application has been reported up until now. Technically, ethanol is a primary metabolite, and its production is tightly associated with

yeast growth. When yeast cells are immobilized by supporting materials, particularly by gel entrapment, cell growth is compromised significantly by the physical constraint, making immobilized cells unlikely productive compared with free yeast cells. Economically, the extra cost of the preparation of immobilized cells at large scales is very high, and the contamination of the supporting materials to the by-products including yeast biomass and feedstock residues to be used as animal feed is unacceptable by the industry. All of these have attributed to the incompetent utilization of immobilized cells for ethanol fermentation (Bai et al. 2008).

When cells self-flocculate/aggregate together to form particles with suitable size distributions, they can be immobilized within fermentors without consumption of any supporting materials, and all disadvantages associated with yeast cells immobilized by supporting materials can be overcome. Recently, this technology was commercialized in fuel ethanol production (Zhao and Bai 2009). More significantly, flocs developed by the self-flocculation of yeast cells might provide close contact for individual cells to benefit their communication and coordination under stressful conditions, and research progress supported such an expectation (Table 2) (Lei et al. 2007), which can be used as a new strategy for yeast cells to overcome stressful conditions.

Table 2 Impact of the self-flocculation of yeast cells on ethanol tolerance and membrane composition

				PI	*PE*	*PC*		
d, μm	*X*, g(DCW)/L	Cell viability,%	*P*, cm/h	μg/g(DCW)			*E*, g/L	*Y*,%
100	10.78	3.5	3.5×10^{-7}	511.7	432.0	363.4	113.0	78.3
200	10.03	26.7	2.9×10^{-7}	857.1	535.0	808.0	117.2	81.2
300	10.07	48.8	1.5×10^{-7}	1429.1	1386.8	413.1	123.4	85.5
400	10.32	37.6	2.0×10^{-7}	1014.6	770.8	96.3	118.8	82.3

The average size of yeast flocs (*d*) was detected online by the focused beam reflectance measurement (FBRM) system, and cell viability was evaluated after yeast flocs were treated by ethanol shock. *X*, *E*, and *Y* biomass, ethanol concentration, and ethanol yield detected at the end of the fermentation; *P* plasma membrane permeability, and *PI*, *PE*, and *PC* phosphoinositol, phosphoethanolamine, and phosphocholine, respectively

5.3 Consecutive Batch Fermentation

Based on the self-flocculation of yeast cells, an innovative process of consecutive batch fermentation was developed for ethanol fermentation under VHG conditions (Li et al. 2009). In this system, a high-cell density was achieved for ethanol fermentation. In the meantime, yeast flocs were separated automatically from the fermentation broth by sedimentation at the end of each batch, so the condensed yeast slurry remained can be applied as inoculums to quickly start the next batch fermentation. The basic idea for such a process design is to alleviate ethanol inhibition in yeast cells by reducing the fermentation time and duration of yeast

cells exposed to high ethanol concentration since the osmotic impact from the VHG medium was approved to be negligible for the self-flocculating yeast.

It seems that the shorter the fermentation time is, the less the ethanol inhibition in yeast flocs could be. However, it was demonstrated that the ethanol productivity also affected the variability of yeast cells and their ethanol fermentation performance (Li et al. 2009), which should be controlled properly in order to maintain suitable cell variability for efficient ethanol fermentation for more batches. Theoretically, this process can be repeated unlimitedly if yeast flocs are purged at the end of each batch to keep a dynamic balance between cell growth and death during the fermentation.

5.4 Process Oscillation

Sustained oscillations of residual sugar, ethanol, and biomass concentrations were observed for continuous ethanol fermentation under VHG conditions. Mechanisms triggering this phenomenon were proposed to be the inhibition of high ethanol concentration accumulated within the fermentation system and the lag response of yeast cells to this stressful condition. Studies using packed bioreactors to immobilize yeast cells and improve their ethanol tolerance as well as the metabolic flux analysis for yeast cells under oscillatory conditions supported this hypothesis (Bai et al. 2004, 2009; Shen et al. 2009, 2010).

It is reasonable for yeast cells to deal with the environmental stress by oscillatory behavior instead of steady state that is prevailing for continuous ethanol fermentation under normal gravity conditions with ethanol concentration $<12\%$ (v/v), as commonly adopted by most laboratory studies. Under oscillatory conditions, sugar and ethanol concentrations change periodically in reverse directions due to their coupling characteristics: The more sugar left, the less ethanol produced, and vice versa. Since osmotic stress from sugar is not a major problem for industrial yeast under continuous ethanol fermentation conditions, the impact of ethanol inhibition can be alleviated periodically through this kind of oscillation, which provides yeast cells opportunities to recover from stressful conditions and be better prepared for the next round of stress attack, and thus, higher ethanol concentration and lower residual sugar could be achieved (Table 3).

Residual sugars must be controlled at extremely low levels in industry, and relatively constant ethanol concentration is also required for the fermentation broth to be processed by distillation. Therefore, the whole ethanol fermentation system cannot be operated at oscillatory conditions. Instead, some tanks in front of the system can be operated at oscillatory state to improve stress tolerance of yeast cells as well as their ethanol productivity, which is attenuated gradually as ethanol fermentation is carried out with the flow of the fermentation broth through the remaining tanks. Thus, steady state is achieved within the last one or two tanks for constant ethanol concentration and required residual sugars in the mature broth, which presents a good example that combines systems biology of yeast cells and

Table 3 Continuous VHG ethanol fermentation operated at oscillatory and steady states

	Oscillatory state		Steady state	
	Glucose, g/L	Ethanol, g/L	Glucose, g/L	Ethanol, g/L
CSTR	112.0	72.8	128.0	65.1
TB 1	27.0	114.3	58.0	99.8
TB 2	18.0	119.8	47.0	105.7
TB 3	11.0	122.9	37.0	110.6
TB 4	5.0	125.4	25.0	115.6

The fermentation system composed of a stirred tank bioreactor (CSTR), followed by four tubular bioreactors (TB 1–4), was operated at the dilution rate of 0.04 h^{-1} by feeding the medium containing 280 g/L glucose, supplemented with 5 g/L yeast extract and 3 g/L peptone. The glucose and ethanol concentrations were average values for comparison

industrial process engineering together to address stress impact exerted on yeast cells under VHG fermentation conditions.

6 Conclusions and Perspectives

The HG/VHG fermentations save energy consumption for both ethanol distillation and the treatment of distillage discharged from the distillation system. Since ethanol concentration achieved during the fermentation of the hydrolysate of lignocellulosic biomass is usually <12% (v/v), the toxic effect of the harmful by-products released during the pretreatment of the feedstock instead of ethanol inhibition will be the major stress exerted on yeast. For ethanol production from starch-based feedstocks, ethanol inhibition in yeast cells is a major concern because much higher ethanol concentration can be achieved; while osmotic stress can be prevented effectively by the SSF process. When sugar-based feedstocks such as molasses are used, osmotic effect may exert on yeast cells, which affects cell growth and ethanol fermentation to certain degree, particularly at the early stage of the fermentation. Also, high temperature is always preferred in industry since the fermentation system operated at elevated temperature can be cooled down by regular cooling water with low cost rather than by chilled water which demands additional capital investment and energy consumption. Thus, the synergistic effect of high temperature and ethanol inhibition may further complicate the industrial fermentation system.

Most studies in stress response of yeast cells are using laboratory strains and media chemically defined or semi-defined with yeast extract and peptone supplemented rather than industrial strains and complex media from various feedstocks. In addition, stresses exerted on yeast cells in laboratory research are generally short period of shocks while sustained and multiple stresses are exerted on yeast cells under industrial conditions, particularly with continuous fermentation systems, which make experimental results, mechanistic analysis, and conclusions developed correspondingly more scientifically significant but limited in practice. For

example, osmotic stress has been intensively studied but will never occur with ethanol fermentations from starch-based feedstocks and lignocellulosic biomass, regardless of how much sugar in the form of starch or cellulose is contained in the media, since the well-established SSF process in the industry makes sugars released from starch or cellulose fermented immediately by yeast cells without significant accumulation within the fermentation system. Therefore, research under ethanol fermentation conditions mimic to industrial production should be applied, and industrial strains are recommended to be used as hosts for genetic manipulations.

Acknowledgment The assistance of Dr. Z. Lewis Liu is gratefully acknowledged. Without his great effort and valuable comments, it would not have been possible for us to make the chapter accommodated to the theme of this book.

References

Aiba S, Shoda M, Nagatani M (1968) Kinetics of product inhibition in alcohol fermentation. Biotechnol Bioeng 10:845–864

Aldiguier AS, Alfenore S, Cameleyre X, Goma G, Uribelarrea JL, Guillouet SE, Molina-Jouve C (2004) Synergistic temperature and ethanol effect on *Saccharomyces cerevisiae* dynamic behavior in ethanol bio-fuel production. Bioprocess Biosyst Eng 26:217–222

Alexander MR, Tyers M, Perret M, Craig BM, Fang KS, Gustin MC (2001) Regulation of cell cycle progression by Swe1p and Hog1p following hypertonic stress. Mol Biol Cell 12:53–62

Arcay-Ledezma GJ, Slaughter JC (1984) The response of *Saccharomyces cerevisiae* to fermentation under carbon dioxide pressure. J Inst Brew 90:81–84

Auesukaree C, Damnernsawad A, Kruatrachue M, Pokethitiyook P, Boonchird C, Kaneko Y, Harashima S (2009) Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. J Appl Genet 50:301–310

Bai FW, Chen LJ, Anderson WA, Moo-Young M (2004) Parameter oscillations in high gravity medium continuous ethanol fermentation and their attenuation on multi-stage packed column bioreactor system. Biotechnol Bioeng 88:558–566

Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 26:89–105

Bai FW, Ge XM, Anderson WA, Moo-Young M (2009) Parameter oscillation attenuation and mechanism exploration for continuous VHG ethanol fermentation. Biotechnol Bioeng 102:112–121

Belli G, Gari E, Aldea M, Herrero E (2001) Osmotic stress causes a G1 cell cycle delay and downregulation of Cln3/Cdc28 activity in *Saccharomyces cerevisiae*. Mol Microbiol 39:1022–1035

Bösl B, Grimminger V, Walter S (2006) The molecular chaperone Hsp104-Amolecular machine for protein disaggregation. J Struct Biol 156:139–148

Cakar ZP, Seker UOS, Tamerler C, Sonderegger M, Sauer U (2005) Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res 5:569–578

Casey GP, Ingledew WM (1986) Ethanol tolerance in yeasts. Crit Rev Microbiol 13:219–280

Devantier R, Pedersen S, Olsson L (2005a) Characterization of very high gravity ethanol fermentation of corn mash: effect of glucoamylase dosage, pre-saccharification and yeast strain. Appl Microbiol Biotechnol 68:622–629

Devantier R, Scheithauer B, Villas-Bôas SG, Pedersen S, Olsson L (2005b) Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. Biotechnol Bioeng 90:703–714

Ferreira C, Lucas C (2007) Glucose repression over *Saccharomyces cerevisiae* glycerol/H^+ symporter gene *STL1* is overcome by high temperature. FEBS Lett 581:1923–1927

Ferreira C, von Voorst F, Martins A, Neves L, Oliveira R, Kielland-Brandt MC, Lucas C, Brandt A (2005) A member of the sugar transporter family, Stl1p is the glycerol/H^+ symporter in *Saccharomyces cerevisiae*. Mol Biol Cell 16:2068–2076

Gauci VJ, Beckhouse AG, Lyons V, Beh EJ, Rogers PJ, Dawes IW, Higgins VJ (2009) Zinc starvation induces a stress response in *Saccharomyces cerevisiae* that is mediated by the Msn2p and Msn4p transcriptional activators. FEMS Yeast Res 9:1187–1195

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807

Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2007) Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. J Biotechnol 131:34–44

Hohmann S (2009) Control of high osmolarity signaling in the yeast *Saccharomyces cerevisiae*. FEBS Lett 583:4025–4029

Ingledew WM (2003) Alcohol production by *Saccharomyces cerevisiae*: a yeast primer. In: Jacques K, Lyons TP, Kelsall DR (eds) The alcohol textbook. Nottingham University Press, Nottingham, pp 49–87

Jung YJ, Park HD (2005) Antisense-mediated inhibition of acid trehalase (*ATH1*) gene expression promotes ethanol fermentation and tolerance in *Saccharomyces cerevisiae*. Biotechnol Lett 27:1855–1859

Kayingo G, Kilian SG, Prior BA (2001) Conservation and release of osmolytes by yeasts during hypo-osmotic stress. Arch Microbiol 177:29–35

Lei JJ, Zhao XQ, Ge XM, Bai FW (2007) Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distributions. J Biotechnol 131:270–275

Li F, Zhao XQ, Ge XM, Bai FW (2009) An innovative consecutive batch fermentation process for very high gravity ethanol fermentation with self-flocculating yeast. Appl Microbiol Biotechnol 84:1079–1086

Lindquist S, Kim G (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. Proc Natl Acad Sci USA 93:5301–5306

Liu ZL, Blaschek HP (2010) Biomass conversion inhibitors and in situ detoxification. In: Vertès AA, Qureshi N, Blaschek HP, Yukawa H (eds) Biomass to biofuels: strategies for global industries. Blackwell Publishing Ltd., Oxford, pp 233–259

Ma M, Liu ZL (2010) Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol 10:169. doi:10.1186/1471-2180-10-169

Magasanik B, Kaiser CA (2002) Nitrogen regulation in Saccharomyces cerevisiae. Gene 290:1–18

Mensonides FIC, Schuurmans JM, de Mattos MJT, Hellingwerf KJ, Brul S (2002) The metabolic response of *Saccharomyces cerevisiae* to continuous heat stress. Mol Biol Rep 29:103–106

Modig T, Granath K, Adler L, Liden G (2007) Anaerobic glycerol production by *Saccharomyces cerevisiae* strains under hyperosmotic stress. Appl Microbiol Biotechnol 75:289–296

Narendranath NV (2003) Bacterial contamination and control in ethanol production. In: Jacques KA, Lyons TP, Kelsall DR (eds) The alcohol textbook. Nottingham University Press, Nottingham, pp 287–298

O'Rourke SM, Herskowitz I (2004) Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. Mol Biol Cell 15:532–542

O'Rourke SM, Herskowitz I, O'Shea EK (2002) Yeast go to the whole HOG for the hyperosmotic response. Trends Genet 18:405–412

Parawira W, Tekere M (2011) Biological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit Rev Biotechnol 31:20–31

Petri R, Schmidt-Dannert C (2004) Dealing with complexity: evolutionary engineering and genome shuffling. Curr Opin Biotechnol 15:298–304

Pham TK, Wright P (2008a) Proteomic analysis of calcium alginate-immobilized *Saccharomyces cerevisiae* under high-gravity fermentation conditions. J Proteome Res 7:515–525

Pham TK, Wright P (2008b) The proteomic response of *Saccharomyces cerevisiae* in very high glucose conditions with amino acid supplementation. J Proteome Res 7:4766–4774

Pienkos PT, Zhang M (2009) Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. Cellulose 16:743–762

Poole K, Walker ME, Warren T, Gardner J, McBryde C, de Barros-Lopes M, Jiranek V (2009) Proline transport and stress tolerance of ammonia-insensitive mutants of the *PUT4*-encoded praline-specific permease in yeast. J Gen Appl Microbiol 55:427–439

Posas F, Saito H (1998) Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J 17:1385–1394

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell 86:865–875

Postmus J, Canelas AB, Bouwman J, Bakker BM, van Gulik W, de Mattos MJT, Brul S, Smits GJ (2008) Quantitative analysis of the high temperature-induced glycolytic flux increase in *Saccharomyces cerevisiae* reveals dominant metabolic regulation. J Biol Chem 283:23524–23532

Rosamond LN, Adam L, Marshall B, Walter PF, Joanne G, Scott R, Kenneth C (2007) The ripple effect: biofuels, food security, and the environment. Environment 49:30–43

Schirmer EC, Homann OR, Kowal AS, Lindquist S (2004) Dominant gain-of-function mutations in Hsp104p reveal crucial roles for the middle region. Mol Biol Cell 15:2061–2072

Service RF (2007) Biofuel researchers prepare to reap a new harvest. Science 315:1488–1491

Shen Y, Zhao XQ, Ge XM, Bai FW (2009) Metabolic flux and cell cycle analysis indicating new mechanism underlying process oscillation in continuous ethanol fermentation with *Saccharomyces cerevisiae* under VHG conditions. Biotechnol Adv 27:1118–1123

Shen Y, Ge XM, Bai FW (2010) Application of oscillation for efficiency improvement of continuous ethanol fermentation with *Saccharomyces cerevisiae* under very-high-gravity conditions. Appl Microbiol Biotechnol 86:103–108

Siderius M, van Wuytswinkel O, Reijenga KA, Kelders M, Mager WH (2000) The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. Mol Microbiol 36:1381–1390

Sonderegger M, Sauer U (2003) Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol 69:1990–1998

Stanley D, Chambers PJ, Stanley GA, Borneman A, Fraser S (2010) Transcriptional changes associated with ethanol tolerance in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 88:231–239

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biological applications. Appl Microbiol Biotechnol 81:211–223

Takagi H, Takaoka M, Kawaguchi A, Kubo Y (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71:8656–8662

Tamás MJ, Luyten K, Sutherland FC, Hernandez A, Albertyn J, Valadi H, Li H, Prior BA, Kilian SG, Ramos J, Gustafsson L, Thevelein JM, Hohmann S (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol 31:1087–1104

Tatebayashi K, Tanaka K, Yang HY, Yamamoto K, Matsushita Y, Tomida T, Imai M, Saito H (2007) Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. EMBO J 26:3521–3533

Teixeira MC, Raposo LR, Mira NP, Lourenço AB, Sá-Correia I (2009) Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. Appl Environ Microbiol 75:5761–5772

Thomas KC, Hynes SH, Ingledew WM (1995) Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. Process Biochem 31:321–331

van Vleet JH, Jeffries TW (2009) Yeast metabolic engineering for hemicellulosic ethanol. Curr Opin Biotechnol 20:300–306

Xue C, Zhao XQ, Bai FW (2010) Effect of the size of yeast flocs and zinc supplementation on continuous ethanol fermentation performance and metabolic flux distribution under very high concentration conditions. Biotechnol Bioeng 105:935–944

Yamamoto N, Maeda Y, Ikeda A, Sakurai H (2008) Regulation of thermotolerance by stress-induced transcription factors in *Saccharomyces cerevisiae*. Eukaryot Cell 7:783–790

Yazawa H, Iwahashi H, Uemura H (2007) Disruption of *URA7* and *GAL6* improves the ethanol tolerance and fermentation capacity of *Saccharomyces cerevisiae*. Yeast 24:551–560

Yoshikawa K, Tanaka T, Furusawa C, Nagahisa K, Hirasawa T, Shimizu H (2009) Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in *Saccharomyces cerevisiae*. FEMS Yeast Res 9:32–44

Zhao XQ, Bai FW (2009) Yeast flocculation: new story in fuel ethanol production. Biotechnol Adv 27:849–856

Zhao XQ, Xue C, Ge XM, Yuan WJ, Wang JY, Bai FW (2009) Impact of zinc supplementation on the improvement of ethanol tolerance and yield of self-flocculating yeast in continuous ethanol fermentation. J Biotechnol 139:55–60

Improving Biomass Sugar Utilization by Engineered *Saccharomyces cerevisiae*

Akinori Matsushika, Z. Lewis Liu, Shigeki Sawayama, and Jaewoong Moon

Contents

1 Introduction 138
2 Xylose Metabolic Pathways 139
3 DNA Recombination of Xylose Reductase and Xylitol Dehydrogenase 139
3.1 Challenges for Improving Xylose Utilization 143
3.2 Improvement of the XR/XDH Pathway 144
4 Genetic Engineering of Xylose Isomerase 145
4.1 Improvement of Xylose Isomerase Pathway 145
4.2 Evolutionary Engineering 146
5 Xylose Transporters 147
5.1 Diauxic Lag 147
5.2 Functions of Sugar Transporter 147
5.3 Applications on *S. cerevisiae* 148
5.4 New Yeast Xylose Transporters 150
5.5 Enhancing Pentose Utilization Using Systems Biology 150
6 Molecular Mechanisms of Xylose Utilization 151
7 Conclusion and Perspectives 152
References 153

Abstract The efficient utilization of all available sugars in lignocellulosic biomass, which is more abundant than available commodity crops and starch, represents one of the most difficult technological challenges for the production of bioethanol. The well-studied yeast *Saccharomyces cerevisiae* has played a traditional and major role in industrial bioethanol production due to its high

A. Matsushika (✉) • S. Sawayama
Biomass Technology Research Center, National Institute of Advanced Industrial Science and Technology, 3-11-32 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan
e-mail: a-matsushika@aist.go.jp

Z.L. Liu • J. Moon
National Center for Agricultural Utilization Research, USDA Agricultural Research Service, 1815 North University Street, Peoria, IL 61604, USA

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_6,

fermentation efficiency. Although *S. cerevisiae* can effectively convert hexose sugars, such as glucose, mannose, and galactose, into ethanol, it is limited to utilize pentose sugars, including xylose and arabinose, leading to low ethanol yields from lignocellulosic biomass. Numerous approaches for enhancing the conversion of pentose sugars to ethanol have been examined, particularly those involving metabolically engineered *S. cerevisiae*. In this chapter, recent progress in several promising strategies, including genetic recombination of xylose reductase, xylitol dehydrogenase, and xylose isomerase, genetic engineering and evolutionary engineering, characterization of xylose transporters, and approaches toward understanding of molecular mechanisms for xylose utilization are discussed, with particular focus on xylose-utilizing strains of engineered *S. cerevisiae*.

1 Introduction

The utilization of biofuels, such as bioethanol, is a promising alternative to liquid fossil fuels for reducing both petroleum dependence and the environmental impact of combustion processes. However, further technology development is required for the efficient production of bioethanol from lignocellulosic biomass, such as wood and agricultural residues, to replace the use of starchy biomass that could be alternatively used for food and animal feed. Lignocellulosic biomass, which is the most abundant material in the world, is comprised of cellulose, hemicellulose, and lignin, whose composition varies widely among plant species (Sun and Cheng 2002). Cellulose and hemicellulose can be used as carbohydrate sources to produce ethanol through chemical or enzymatic hydrolysis (saccharification) and fermentation. During hydrolysis, glucose is released from the cellulose component of lignocellulose, while pentose (i.e., xylose and arabinose) and hexose (i.e., glucose, mannose, and galactose) sugars are released from the hemicellulose component. As xylose, the second most abundant monosaccharide in nature following glucose is the dominant pentose sugar in hemicellulose hydrolysates; economically feasible biomass–ethanol fermentation processes require the utilization of this pentose sugar. However, only a few traditional ethanol-producing microorganisms can ferment xylose, albeit with limited efficiency.

In the past two decades, several microorganisms, including yeasts and bacteria (e.g., *Zymomonas mobilis* and *Escherichia coli*), have been engineered to convert xylose to ethanol (Dien et al. 2003; Lin and Tanaka 2006). Among yeasts, *Saccharomyces cerevisiae* has traditionally been used for industrial ethanol production because of its high ethanol productivity, tolerance to ethanol (Taylor et al. 2008) and lignocellulose-derived inhibitory compounds (Olsson and Hahn-Hägerdal 1993; Olsson and Nielsen 2000; Liu et al. 2004, 2008), relative resistance to low pH (a characteristic that reduces contamination by other bacteria), and is generally regarded as safe. Although *S. cerevisiae* does not exhibit many of the limitations encountered with bacteria, as stated above, it is unable to utilize xylose for growth or fermentation. Therefore, metabolic engineering of this promising microorganism

for efficient xylose fermentation has been a major goal of many research groups. To date, many *S. cerevisiae* strains capable of utilizing xylose for ethanol production have been successfully engineered (Table 1). Numerous excellent reviews have addressed the current advances in metabolic engineering of xylose-utilizing strains and factors which affect xylose metabolism in yeasts (Gong et al. 1999; Ho et al. 1999; Jeffries and Shi 1999; Aristidou and Penttilä 2000; Hahn-Hägerdal et al. 2001, 2007a, b; Jeffries and Jin 2004; Jeffries 2006; van Maris et al. 2006, 2007; Chu and Lee 2007; Almeida and Hahn-Hägerdal 2009; Van Vleet and Jeffries 2009; Matsushika et al. 2009c). This chapter focuses on recent advances in the improvement of xylose utilization by engineered strains of *S. cerevisiae* for bioethanol production.

2 Xylose Metabolic Pathways

Xylose is converted to the keto-isomer xylulose through two different pathways in microorganisms. In xylose-utilizing bacteria (e.g., *E. coli* and *Streptomyces* sp.), xylose is directly isomerized to xylulose by xylose isomerase (XI). Xylulokinase (XK) then phosphorylates xylulose to yield xylulose 5-phosphate (X5P), which is further metabolized through the pentose phosphate pathway (PPP) and glycolysis (Fig. 1). In contrast, most fungi and xylose-fermenting yeasts (e.g., *Scheffersomyces* (*Pichia) stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*) convert xylose to xylulose by two oxidoreductases that require the cofactors NAD(P)H/NAD(P)$^+$. In this pathway, NAD(P)H-linked xylose reductase (XR) initially reduces xylose to xylitol, which is then oxidized to xylulose by NAD$^+$-linked xylitol dehydrogenase (XDH). Subsequent phosphorylation of xylulose is followed by X5P metabolism and glycolysis, as occurs in bacteria.

3 DNA Recombination of Xylose Reductase and Xylitol Dehydrogenase

As *S. cerevisiae* is only able to metabolize xylulose at a minimal rate (Wang and Schneider 1980; Hsiao et al. 1982), the conversion of xylose to xylulose is a critical target for the metabolic engineering of an efficient xylose-utilizing *S. cerevisiae* strain. Anaerobic ethanol fermentation from xylose using recombinant *S. cerevisiae* strains has predominantly been accomplished by heterologous expression of the *S. stipitis XYL1* and *XYL2* genes, which encode XR (*Ps*XR) and XDH (*Ps*XDH) (Kötter and Ciriacy 1993; Tantirungkij et al. 1993), respectively.

Table 1 A survey of representative *Saccharomyces* strains with improved xylose utilization through genetic engineering and directed adaptation

Strain	Background	Condition	Sugar (g L^{-1})	Medium	Ethanol (g g^{-1})	Xylitol (g g^{-1})	Reference
pRD1	*XYL1*, *XYL2*	Aerobic batch	Xylose 20	cm	0.07	0.46	Kötter and Ciriacy (1993)
1400 (pLNH32)	*XYL1*, *XYL2*, *XKS1*	Fermentative batch	Glucose 90, xylose 40	cm	0.46	0.17	Ho et al. (1998)
1400 (pLNH32)	*XYL1*, *XYL2*, *XKS1*	Fermentative batch	Xylose 50	cm	0.30	0.08	Ho et al. (1998)
TMB3001	*XYL1*, *XYL2*, *XKS1*	Anaerobic continuous	Glucose 15, xylose 5	sm	0.3	0.03	Eliasson et al. (2000b)
H158-pXks	*XYL1*, *XYL2*, *XKS1*	Fermentative batch	Xylose 80	cm	0.27	0.03	Johansson et al. (2001)
H1691	*XYL1*, *XYL2*, *XKS1*	Aerobic batch	Xylose 50	sm	0.06	0.06	Toivari et al. (2001)
H1691	*XYL1*, *XYL2*, *XKS1*	Microaerobic batch	Xylose 50	sm	0.12	0.42	Toivari et al. (2001)
H1691	*XYL1*, *XYL2*, *XKS1*	Anaerobic batch	Xylose 50	sm	0.09	0.41	Toivari et al. (2001)
TMB3102	*XYLA*, Δ*gre3*	Anaerobic batch	Xylose 50	sm	0.22	0.31	Träff et al. (2001)
TMB3008	*XYL1*, *XYL2*, *XKS1*, Δ*gnd1*	Anaerobic batch	Xylose 50	sm	0.38	0.13	Jeppsson et al. (2002)
TMB3255	*XYL1*, *XYL2*, *XKS1*, Δ*zwf1*	Anaerobic batch	Xylose 50	sm	0.41	0.05	Jeppsson et al. (2002)
A4	*XYL1*, *XYL2*, *XKS1*	Aerobic batch	Glucose 50, xylose 50	sm	0.27	0.27	Zaldivar et al. (2002)
H2684	*XYL1*, *XYL2*, *XKS1*, *GDP1*, Δ*zwf1*	Anaerobic batch	Xylose 50	sm	0.34	0.34	Verho et al. (2003)
TMB3001C1	Adaptation select of TMB3001 (heterozygous populations)	Anaerobic batch	Xylose 10	sm	0.24	0.32	Sonderegger and Sauer (2003)
TMB3400	*XYL1* ,*XYL2* ,*XKS1*, random mutagenesis	Anaerobic batch	Xylose 20	sm	0.18	0.25	Wahlbom et al. (2003a)
FPL-YSX3	*XYL1*, *XYL2*, *XYL3*	Aerobic fermentation	Xylose 40	sm	0.12	0.27	Jin et al. (2003)
FPL-YSX3P	Respiration-deficient *XYL1*, *XYL2*, *XYL3*	Aerobic fermentation	Xylose 40	cm	0.29	0.46	Jin et al. (2004)
424A(LNH-ST)	*XYL1*, *XYL2*, *XKS1*	Fermentative batch	Glucose 70, xylose 40	cm	0.41	0.11	Sedlak and Ho (2004)
RW 202-AFX	Adaptation select of RWB202 (*XYLA*)	Anaerobic batch	Xylose 20	sm	0.42	0.021	Kuyper et al. (2004)

TMB3050	Adaptation select of TMB3045 (*XYLA*, *XKS1*,*TAL1*,*TKL1*, *RPI1*, *RPE1*, Δ*gre3*)	Oxygen-limited fermentation	Xylose 50	sm	0.29	0.23	Karhumaa et al. (2005)
RWB 218	Adaptation select of RWB217 (*XYLA*, *XKS1*, *TAL1*,*TKL1*, *RKI1*, *RPE1*, Δ*gre3*)	Anaerobic batch	Xylose 20	sm	0.41	0.001	Kuyper et al. (2005b)
TMB3270	1 × *XYL1* (K270M), *XYL2*, *XKS1*	Anaerobic batch	Xylose 50	sm	0.36	0.17	Jeppsson et al. (2006)
TMB3271	2 × *XYL1* (K270M), *XYL2*, *XKS1*	Anaerobic batch	Xylose 50	sm	0.31	0.09	Jeppsson et al. (2006)
TMB3057	*XYL1*, *XYL2*, *XKS1*, *TAL1*, *TKL1*, *RKI1*, *RPE1*, Δ*gre3*	Anaerobic batch	Xylose 50	sm	0.33	0.22	Karhumaa et al. (2007b)
TMB3066	*XYLA*, *XKS1*, *TAL1*, *TKL1*, *RKI1*, *RPE1*, Δ*gre3*	Anaerobic batch	Xylose 50	sm	0.43	0.04	Karhumaa et al. (2007b)
TMB3400	*XYL1*, *XYL2*, *XKS1*, random mutagenesis	Anaerobic batch	Xylose 50	sm	0.34	0.29	Karhumaa et al. (2007b)
DR PHO13 (pRS314-X123)	*XYL1*, *XYL2*, *XYL3*, Δ*pho13*	Aerobic batch	Xylose 40	sm	0.25	0.04	Ni et al. (2007)
BP10001	*XYL1* (K274-N276D), *XYL2*, *XKS1*	Oxygen-limited	Xylose 20	sm	0.34	0.17	Petschacher and Nidetzky (2008)
BP10001	*XYL1* (K274-N276D), *XYL2*, *XKS1*	Anaerobic batch	Xylose 20	sm	0.34	0.19	Petschacher and Nidetzky (2008)
ADAP8	Adaptation select of INVSc1/ pRS406XKS/pILSUT1/ pWOXYLA (*XYLA*, *XKS1*, *SUT1*)	Anaerobic batch	Xylose 20	cm	0.35	0.16	Madhavan et al. (2009b)
MA-R5	*XYL1*, *XYL2* (D207A, I208R, F209S, N211R), *XKS1*	Anaerobic batch	Xylose 45	cm	0.37	0.038	Matsushika et al. (2009b)
NRRL Y-50463	YXI^{syn}, *XUT4*, *XUT6*, *XKS1*,*XYL2*	Anaerobic batch	Glucose 50, xylose 50	sm	0.39	0.075	Ma et al. unpublished

cm complex medium, *sm* synthetic medium

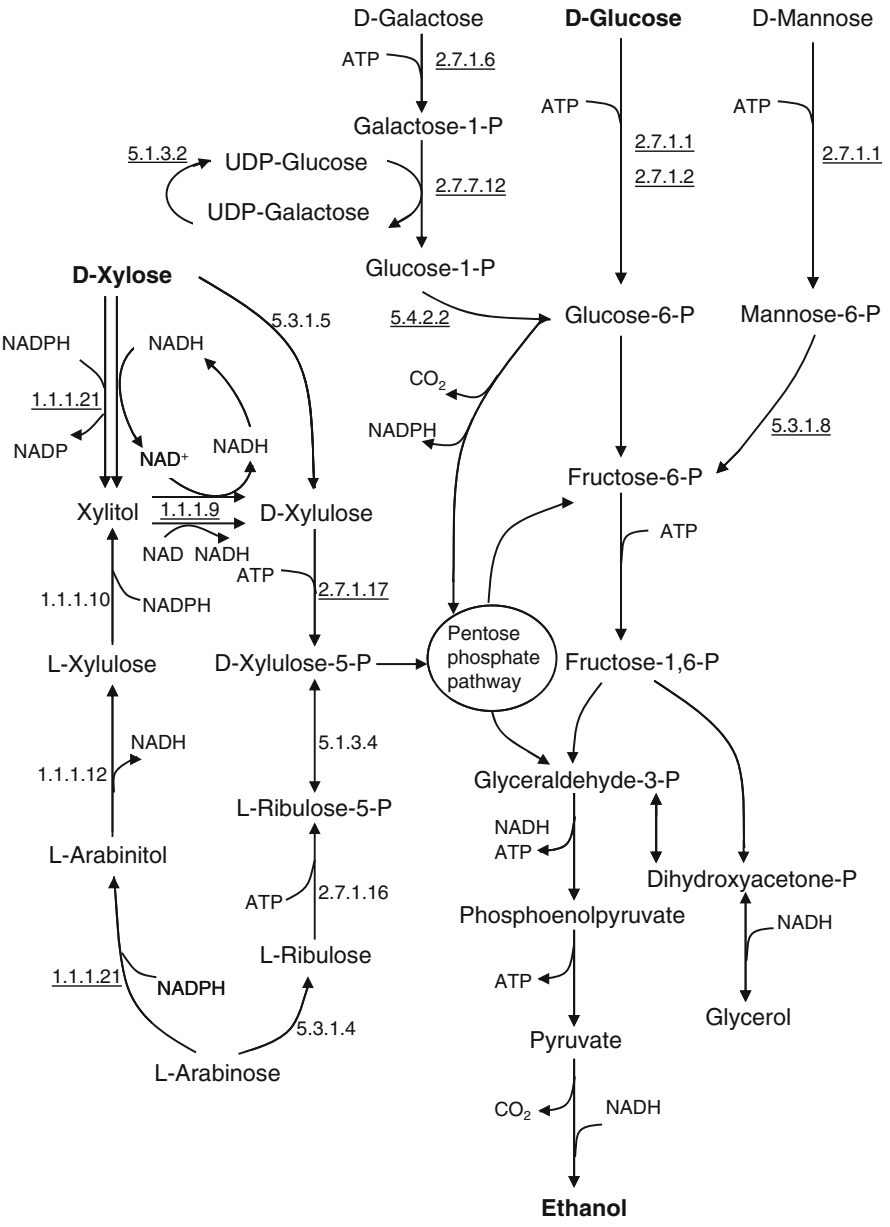

Fig. 1 An overview of catabolic pathways for engineered *Saccharomyces cerevisiae* in utilization of major hexoses including glucose, galactose, and mannose, and pentoses including xylose and arabinose for ethanol production. In the diagram, *underlined* EC numbers represent endogenous enzymes, and those in normal cases indicate exogenous origin or introduced to the yeast. Enzyme-encoding genes and EC numbers are presented in parentheses as follows: hexokinase (*HXK1*/*HXK2, 2.7.1.1*); glucokinase (*GLK1, 2.7.1.2*); galactokinase (*GAL1, 2.7.1.6*); galactose-1-phosphate uridylyltransferase (*GAL7*, *2.7.7.12*); UDP-glucose 4-epimerase (*GAL10*, *5.1.3.2*);

3.1 Challenges for Improving Xylose Utilization

Although expression of both *Ps*XR and *Ps*XDH permits the growth of *S. cerevisiae* on xylose, such engineered strains produce little ethanol and accumulate a considerable amount of xylitol as a by-product, limiting their application in industrial bioprocesses. One of the main reasons for this poor ethanol production is an intracellular redox imbalance resulting from differences in coenzyme specificities between the mainly NADPH-dependent XR and strictly NAD^+-dependent XDH (Bruinenberg et al. 1983; Kötter and Ciriacy 1993).

In addition to redox imbalance, poor ethanol yields from *S. cerevisiae* are ascribed to low levels of endogenous XK activity, which leads to slow xylulose consumption (Chang and Ho 1988; Deng and Ho 1990). However, overexpression of the *XKS1* gene encoding XK from *S. cerevisiae* (*Sc*XK) improves ethanol production from xylose (Ho et al. 1998; Eliasson et al. 2000b; Toivari et al. 2001), although xylitol remains a major by-product. Thus, controlling the specific expression of XR, XDH, and XK is essential for effective utilization of xylose, as a higher level of XDH relative to XR decreases the xylitol yield (Walfridsson et al. 1997; Eliasson et al. 2001; Jin and Jeffries 2003; Karhumaa et al. 2007a; Matsushika et al. 2009a), while high activity of both XR and XDH is important for xylose fermentation (Jeppsson et al. 2003; Karhumaa et al. 2007a; Matsushika and Sawayama 2008). In addition, recent studies have demonstrated that only finely tuned overexpression of XK in *S. cerevisiae* leads to improved xylose fermentation (Rodriguez-Pena et al. 1998; Johansson et al. 2001; Jin et al. 2003; Matsushika and Sawayama 2008).

Optimizing fermentation conditions, such as temperature, pH, growth substrates, and initial cell concentrations, is important for obtaining the maximum rate and yield of ethanol production from xylose. For instance, starting the fermentation with a highly concentrated inoculum of metabolically engineered *S. cerevisiae* strains can improve the rate of xylose utilization and ethanol production (Zhong et al. 2009; Matsushika and Sawayama 2010). In recombinant *S. cerevisiae* strains generated to date, however, the rate of ethanol production from xylose is considerably lower than that from glucose. The difference in ethanol production between these two substrates may be related to the use of xylose as a nonfermentable carbon source (Salusjärvi et al. 2003, 2008; Jin et al. 2004; Souto-Maior et al. 2009).

The uptake of xylose by *S. cerevisiae* is considered to be one of the main rate-limiting steps of xylose metabolism due to the lack of xylose-specific transporters in this yeast. The uptake of xylose proceeds through hexose transporters encoded by the *HXT* gene family (Kruckeberg 1996), albeit with significantly lower affinity

Fig. 1 (continued) phosphoglucomutase (*GAL5*/*PGM2*, *5.4.2.2*); hexokinase I (*HXK1*, *2.7.1.1*); mannose-6-phosphate isomerase (*PMI40*, *5.3.1.8*); xylose reductase/aldose (*GRE3*/*xyl1*, *1.1.1.21*); xylitol dehydrogenase (*XYL2*/*xyl2*, *1.1.1.9*); xylulokinase (*XKS1*/*xyl3*, *2.7.1.17*); xylose isomerase (YXI^{syn}/*xylA*, *5.3.1.5*); arabinitol 4-dehydrogenase (*lad1*, *1.1.1.12*); L-xylulose reductase (*lxr1*, *1.1.1.10*); L-arabinose isomerase (*araA*, *5.3.1.4*); L-ribulokinase (*araB*, *2.7.1.16*); and L-ribulose-5-phosphate 4-epimerase (*araD*, *5.1.3.4*) (This figure is reprinted from Liu et al. 2008b)

compared to glucose (Kötter and Ciriacy 1993). The transport of xylose is thus competitively inhibited by glucose, and in the case of mixed sugar substrates, xylose is typically consumed only after the depletion of glucose (van Zyl et al. 1993; Sedlak and Ho 2004). Another factor limiting ethanol production from xylose in *S. cerevisiae* is the lower activity of the PPP compared to that in other species of yeast (Gancedo and Lagunas 1973; Fiaux et al. 2003), as this pathway also affects the rates of xylulose conversion. This finding partially explains why *S. cerevisiae* almost exclusively produces ethanol from hexose sugars.

3.2 Improvement of the XR/XDH Pathway

Recombinant *S. cerevisiae* strains capable of utilizing the XR/XDH pathway excrete substantial amounts of xylitol as a by-product, thereby decreasing ethanol yields. This finding is mainly ascribed to differences in coenzyme specificity between the predominantly NADPH-dependent XR and strictly NAD^+-dependent XDH, as described above. Under anaerobic conditions, the poor recycling of NAD^+ causes an intracellular redox imbalance in *S. cerevisiae* (Bruinenberg et al. 1983; Kötter and Ciriacy 1993). Therefore, altering the coenzyme specificity of XR and/or XDH by protein engineering is an attractive approach for reducing xylitol production and enhancing ethanol yield using recombinant *S. cerevisiae*.

Several research groups have engineered *S. cerevisiae* strains to express mutated XR/XDH enzymes from various yeasts. For example, Nidetzky and coworkers generated several NADH-preferring mutant enzymes of XR from *Candida tenuis* (*Ct*XR), which were then used to generate several *S. cerevisiae* strains (Kavanagh et al. 2002, 2003; Leitgeb et al. 2005; Petschacher and Nidetzky 2005; Petschacher et al. 2005). One of the recombinant strains, *S. cerevisiae* strain BP10001 harboring the K274R-N276D *Ct*XR double mutant, exhibited decreased xylitol formation (Petschacher and Nidetzky 2008). In addition to XR, four $NADP^+$-preferring XDH mutants have also been generated from *Galactocandida mastotermitis* (*Gm*XDH). Two of the *S. cerevisiae* strains, expressing mutated *Gm*XDH in combination with a matching NADPH-preferring *Ct*XR mutant, showed decreased glycerol yield without an increase in ethanol production (Krahulec et al. 2009). Jeppsson et al. (2006) engineered a recombinant *S. cerevisiae* strain that expresses mutated *Ps*XR, which has reduced affinity for NADPH (K270M; Kostrzynska et al. 1998), resulting in enhanced ethanol production accompanied by decreased xylitol formation. Makino and collaborators (Watanabe et al. 2007a, b) also created several strains expressing NADH-preferring *Ps*XR mutants and found that the R276H strain had the most positive effect on xylose fermentation to ethanol. This group also generated several *Ps*XDH mutants with complete reversal of coenzyme specificity toward $NADP^+$ (Watanabe et al. 2005) and demonstrated that expression of a quadruple ARSdR mutant (D207A/I208R/F209S/N211R) in recombinant *S. cerevisiae* achieved increased ethanol and decreased xylitol production (Watanabe et al. 2007c; Matsushika et al. 2008a, b, 2009b). Significantly,

expression of this modified enzyme also increased xylose consumption and ethanol production rates with not only xylose-supplemented artificial medium, but also with lignocellulosic hydrolysate as a substrate (Matsushika et al. 2008b, 2009b).

As another strategy to relieve intracellular redox imbalance, Jeppsson et al. (2002) constructed a *Ps*XR-*Ps*XDH-*Sc*XK – expressing *S. cerevisiae* strain by inactivating the oxidative PPP through the *GND1* and *ZWF1* genes, which encodes NADPH-producing 6-phosphogluconate dehydrogenase (6PGDH) and glucose-6-phosphate dehydrogenase (G6PDH), respectively. Compared to the parent strain, the Δ*gnd1* and Δ*zwf1* mutants decreased xylitol yield, with a corresponding increase in ethanol yield. Using a slightly different approach, Verho et al. (2003) demonstrated that the rate and yield of xylose fermentation to ethanol can be improved by expressing the *Kluyveromyces lactis GDP1* gene (Verho et al. 2002), encoding $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase, and deleting the *ZWF1* gene. In addition, deletion of the *GRE3* gene encoding a strictly NADPH-dependent aldose reductase decreases xylitol excretion by recombinant *S. cerevisiae* (Träff et al. 2001; Träff-Bjerre et al. 2004), which represents a particularly useful strategy for XI-expressing strains, because xylitol inhibits the activity of XI (Yamanaka 1969; Lönn et al. 2003; Kuyper et al. 2005a).

4 Genetic Engineering of Xylose Isomerase

Eukaryotic pathways for xylose metabolism utilize oxidoreductases with different cofactor requirements, yielding a substantial increase in xylitol accumulation. To avoid cofactor imbalance in *S. cerevisiae*, expression of bacterial XI encoded by the *xylA* gene may be a reasonable approach (Walfridsson et al. 1996; Kuyper et al. 2003; Lönn et al. 2003); however, nearly all attempts to express functional XI in this yeast have failed because of reduced or no XI activity (Sarthy et al. 1987; Amore et al. 1989; Moes et al. 1996; Gárdonyi and Hahn-Hägerdal 2003). Unsuccessful heterologous expression in *S. cerevisiae* is speculated as a result of protein misfolding, improper posttranslational modifications, and disulfide bridge formation (Sarthy et al. 1987).

4.1 Improvement of Xylose Isomerase Pathway

Walfridsson et al. (1996) reported the first successful expression of a functional XI from *Thermus thermophilus* in *S. cerevisiae*. Although reduced xylitol excretion had been previously achieved when *T. thermophilus* XI expression was combined with other genetic modifications (Träff et al. 2001), the activity of XI at 30°C was too low to allow fermentation of xylose. In a subsequent attempt at XI overexpression, three cold-adapted XI mutants, which were created by random PCR mutagenesis and expressed individually in a recombinant *S. cerevisiae* strain (Lönn et al. 2002), did not permit ethanol production from xylose at 30°C

(Lönn et al. 2003). Recent studies, however, have reported successful heterologous expression of XI genes from the anaerobic fungi *Piromyces* (Kuyper et al. 2003) and *Orpinomyces* (Madhavan et al. 2009a), as well as bacterial XI from *Clostridium phytofermentans* (Brat et al. 2009), in *S. cerevisiae* at high levels. Further adaptation (Kuyper et al. 2004, 2005b; Madhavan et al. 2009b) and genetic engineering (Kuyper et al. 2005a; Hughes et al. 2009) have been applied for improving xylose fermentation to ethanol. However, growth on xylose and xylose fermentation in an XI-expressing strain was slower than in an XR-XDH-expressing strain, despite the higher yield of ethanol (Karhumaa et al. 2007b). Moreover, XI has only been expressed under strong promoters on multicopy plasmids, indicating that XI-expressing strains tend to be unstable, particularly during continuous cultivation. A recent development by chromosomal integration of a synthesized yeast xylose isomerase gene into tolerant industrial yeast appeared to be stable and promising for engineered yeast strain development using xylose isomerase (Liu et al. 2011; Ma et al. unpublished data). See following sections for more detailed discussions.

4.2 Evolutionary Engineering

Approaches involving natural selection, random mutation, and evolutionary engineering (Sauer 2001) have also been applied to a number of *S. cerevisiae* strains for enhancing xylose fermentation (Sonderegger and Sauer 2003; Wahlbom et al. 2003a; Kuyper et al. 2004, 2005b; Karhumaa et al. 2005; Pitkänen et al. 2005; Madhavan et al. 2009b; Wisselink et al. 2009; Matsushika et al. 2010). As a number of recombinant *S. cerevisiae* strains are often unable to grow anaerobically on xylose alone, these approaches allow the generation of adapted strains that are not only capable of anaerobic growth on xylose as the sole carbon source, but also exhibit improved xylose utilization. Notably, evolutionary engineering approaches, in which xylose-utilizing *S. cerevisiae* strains were adapted to lignocellulosic hydrolysates, have been used to improve tolerance to inhibitors (Liu et al. 2005; Heer and Sauer 2008) and ethanol production (Martín et al. 2007). Microarray, metabolic flux, enzymatic, and metabolite analyses of these evolved strains have provided important comparative information concerning bottlenecks in xylose metabolism (Sonderegger et al. 2004; Wahlbom et al. 2003b; Pitkänen et al. 2005; Bengtsson et al. 2008; Karhumaa et al. 2009); however, in many cases, the observed changes in evolved strains were identical to those observed in earlier metabolically engineered strains.

As robustness and tolerance to inhibitors present in lignocellulosic hydrolysates depend on the strain background (Martín and Jönsson 2003; Brandberg et al. 2004), the selection of a suitable xylose-utilizing *S. cerevisiae* host strain is important for efficient industrial ethanol fermentation of lignocellulose. *S. cerevisiae* strains have varying xylulose conversion abilities (Yu et al. 1995; Eliasson et al. 2000a; Matsushika et al. 2009a, b) due to differences in PPP flux linking the

xylose-to-xylulose conversion pathway to glycolysis (Johansson and Hahn-Hägerdal 2002a). Therefore, evaluation of xylulose conversion ability is a useful engineering strategy for selecting a suitable yeast host strain for fermentation of xylose.

5 Xylose Transporters

For most previous efforts to improve xylose utilization using genetic engineering methods, xylose transporters were not included in recombinant *S. cerevisiae*. However, the importance and necessity of an efficient xylose transport system in a host cannot be underestimated. The lack of such a transport system in *S. cerevisiae* may be a major hurdle, preventing balanced utilization of divergent biomass sugars by the yeast.

5.1 Diauxic Lag

In the presence of glucose, xylose-utilizing enzymes are repressed as mentioned earlier, which effectively inactivates xylose catabolism until glucose is depleted. For mixed-sugar utilization by yeast, including natural pentose-utilizing *S. stipitis* and engineered strains of *S. cerevisiae*, diauxic lag during the sequential consumption of the substrates is a commonly observed practical problem (Slininger et al. 1987; Krishnan et al. 1999; Zaldivar et al. 2002; Kuyper et al. 2005b). Stalling during the transition to xylose may be due in part to oxygen-dependent induction of xylose-specific transporters and enzymes. The diauxy not only causes economic losses by extending fermentation process time, but it also introduces additional contamination opportunities.

5.2 Functions of Sugar Transporter

Sugar transport across the plasma membrane is a necessary first step of carbohydrate utilization. In yeast cells, the uptake of carbohydrates is mediated by a large family of related transporter proteins. Disruption of the transport process hinders biosynthesis by limiting uptake of essential amino acids, sugars, and other essential nutrients. Numerous monosaccharide transporters in yeasts function through facilitated diffusion, which is an energy-independent mechanism that allows substances to freely cross membranes. Other yeast monosaccharide transporters are proton symporters, which typically operate only when the amount of sugar is limited. Proton symporters are energy-consuming systems that are able to transport a monosaccharide against its concentration gradient, coupled to the simultaneous movement of protons. Comprehensive information on function and classification of

sugar transporter proteins is available elsewhere (Saier et al. 2006; http://www.tcdb.org/; http://homes.esat.kuleuven.be/~sbrohee/ytpdb/index.php/Main_Page).

5.3 *Applications on* S. cerevisiae

There are 20 different transporter-related proteins for hexose that have been identified in *S. cerevisiae* (Kruckeberg 1996). Among these proteins, HXT1, HXT2, HXT4, HXT5, HXT7, and GAL2 serve as xylose-transporting proteins (Hamacher et al. 2002; Saloheimo et al. 2007); however, their affinity for xylose is significantly lower than that for glucose, and xylose uptake is strongly inhibited in the presence of glucose (Saloheimo et al. 2007; van Zyl et al. 1993). Most xylose transporters studied to date are from *S. stipitis* since it is a natural xylose-utilizing yeast. Xylose uptake in *S. stipitis* is mediated by at least two transport systems, involving low- and high-affinity proton symporters (Kilian and van Uden 1988; Does and Bisson 1989). The limiting factor of xylose catabolism in this yeast appears to be the uptake of xylose into cells, at least under aerobic conditions (Ligthelm et al. 1988; Kilian and van Uden 1988).

In *S. cerevisiae*, xylose uptake is mediated by a nonspecific hexose transport system and is significantly less efficient than that of glucose. The characterized glucose transporter genes *SUT1*, *SUT2,* and *SUT3* from *S. stipitis* exhibit a much higher affinity for glucose than xylose (Weierstall et al. 1999). The glucose transporters SUT1, SUT2, and SUT3 function via facilitated diffusion, with Km values in the millimolar range. These transporter proteins are also able to transport xylose and other monosaccharides, but with a considerably lower affinity. In addition, culture conditions influence the expression of *SUT1*, *SUT2*, and *SUT3*. For example, the transcription of *SUT1* is strongly induced by glucose and is independent of the oxygen supply, whereas *SUT2* and *SUT3* are only expressed under aerobic conditions and are independent of the carbon source (Weierstall et al. 1999). Under semi-anaerobic conditions, xylose uptake activity was observed in a *S. stipitis* strain with a *sut1* mutation when SUT2 and SUT3 were not expressed, which indicates that additional regulatory systems exist for high-affinity xylose uptake (Weierstall et al. 1999). High (HXT7 and GAL2)- and intermediate (HXT4 and HXT5)-affinity glucose transporters in *S. cerevisiae* also show limited xylose transport potentials (Hamacher et al. 2002; Sedlak and Ho 2004). Notably, expression of *SUT1* in xylose-assimilating *S. cerevisiae* increases both xylose uptake ability and ethanol productivity during xylose fermentation (Katahira et al. 2008).

Heterologous expression of a xylose transporter homologue (TrXLT1) isolated from *Trichoderma reesei* in a *S. cerevisiae* strain lacking the major hexose and galactose transporter genes (*hxt1-7* and *gal2*) led to cell growth on xylose, but not on glucose (Saloheimo et al. 2007). This finding suggests that TrXLT1 possesses xylose-specific activity. In addition, two xylose transporters, *GXF1* and *GXS1* from *Candida intermedia*, an efficient xylose-utilizing yeast, were cloned and characterized for potential enhancing xylose transport in *S. cerevisiae*

Table 2 Xylose transporters characterized for improving xylose uptake and utilization in *Saccharomyces cerevisiae*

Gene	Origin	Type	Substrate (Km, mM)	Reference
SUT1	*S. stipitis*	Facilitator	Glucose (1.5) Fructose (36) Xylose (145)	Weierstall et al. (1999) Runquist et al. (2009a)
SUT2	*S. stipitis*	Facilitator	Glucose (1.1) Xylose (49)	Weierstall et al. (1999)
SUT3	*S. stipitis*	Facilitator	Glucose (0.8) Fructose (49) Xylose (103) Galactose (176)	Weierstall et al. (1999)
HXT1	*S. cerevisiae*	Facilitator	Xylose (880)	Sedlak and Ho (2004) Saloheimo et al. (2007)
HXT2	*S. cerevisiae*	Facilitator	Xylose (260)	Sedlak and Ho (2004) Saloheimo et al. (2007)
HXT4	*S. cerevisiae*	Facilitator	Xylose (170)	Hamacher et al. (2002) Sedlak and Ho (2004) Saloheimo et al. (2007)
HXT5	*S. cerevisiae*	Facilitator	Xylose	Hamacher et al. (2002) Sedlak and Ho (2004)
HXT7	*S. cerevisiae*	Facilitator	Xylose	Hamacher et al. (2002) Sedlak and Ho (2004) Saloheimo et al. (2007)
GAL2	*S. cerevisiae*	Facilitator	Xylose	Hamacher et al. (2002) Sedlak and Ho (2004) Saloheimo et al. (2007)
TrXLT1	*Trichoderma reesei*	Unknown	Xylose	Saloheimo et al. (2007)
GXS1	*Candida intermedia*	Symporter	Glucose (0.2) Xylose (0.4)	Leandro et al. (2006)
GXF1	*Candida intermedia*	Facilitator	Glucose (2) Xylose (49)	Leandro et al. (2006) Runquist et al. (2009, 2010)
At5g59250	*Arabidopsis thaliana*	Unknown	Xylose	Hector et al. (2008) Runquist et al. (2010)
At5g17010	*Arabidopsis thaliana*	Unknown	Xylose	Hector et al. (2008)
An5 (EAA35128)	*Neurospora crassa*	Facilitator	Xylose (176)	Du et al. (2010)
Xyp29(XUT6)	*S. stipitis*	Facilitator	Xylose (56)	Du et al. (2010)
XUT4	*S. stipitis*	Unknown	Xylose	Moon et al. unpublished
XUT5	*S. stipitis*	Unknown	Xylose	Moon et al. unpublished
XUT6	*S. stipitis*	Facilitator	Xylose	Moon et al. unpublished
XUT7	*S. stipitis*	Unknown	Xylose	Moon et al. unpublished
RGT2	*S. stipitis*	Unknown	Xylose	Moon et al. unpublished
SUT4	*S. stipitis*	Unknown	Xylose	Moon et al. unpublished

(Leandro et al. 2006) (Table 2). *GXF1* is a glucose/xylose facilitator (Km 49 mM for xylose), and *GXS1* is a glucose/xylose proton symporter (Km 0.4 mM for xylose), which both displayed higher affinities for glucose than xylose. Coexpression of *GXF1* and *GXS1* in *S. cerevisiae* drastically reduced *GXS1* mRNA levels and consequently, symport activity, suggesting that limiting the expression of high-affinity sugar transporter systems may be a widespread mechanism in yeast whenever their activities are dispensable (Leandro et al. 2008). GXF1 and GXS1 from *C. intermedia* have also been expressed in a xylose-utilizing *S. cerevisiae* strain, which resulted in faster xylose uptake and ethanol production (Runquist et al. 2009a). Recently, two heterologous xylose transporters from *Arabidopsis thaliana* (At5g59250 and At5g17010) were expressed in *S. cerevisiae*, resulting in increased xylose uptake and consumption by 46% and 40%, respectively (Hector et al. 2008). Using *GXS1* from *C. intermedia* as a probe sequence, *An25* from *Neurospora crassa* and *XUT6* from *S. stipitis* were found to function for xylose transport (Du et al. 2010).

5.4 New Yeast Xylose Transporters

Annotations of genome sequence of *S. stipitis* CBS 6054 suggested that putative xylose transporter genes exist in this yeast (Jeffries et al. 2007; http://www.ncbi.nlm.nih.gov/). Although high-affinity xylose transporters were not identified, several new xylose transporter genes, *XUT1, XUT2, XUT3, XUT4, XUT5, XUT6, XUT7*, *SUT4*, *RGT2*, and *HXT2.4* from *S. stipitis* were cloned and expressed in a *S. cerevisiae* strain with tolerance to inhibitors present in lignocellulose hydrolysates (Moon et al. unpublished) (Table 2). The genetically engineered *S. cerevisiae* strains with heterologous *XUT5, XUT7*, and *RGT2* showed high levels of expression under both aerobic and anaerobic conditions. Overexpression of these putative xylose transporter genes in *S. cerevisiae* resulted in higher intracellular xylose accumulations than that in wild-type cells. These transporter genes also enhanced expression of yeast xylose isomerase. Such results suggest these genes encode proteins involved in xylose transport (Moon et al. unpublished data). Further investigations of these candidate genes are expected to facilitate enhanced xylose utilization by engineered *S. cerevisiae*.

5.5 Enhancing Pentose Utilization Using Systems Biology

A significant lesson we learned from genomics is the underestimate of complex interactions of a biological system. Often, a single gene approach is unable to resolve complicated problems such as efficient utilization of divergent biomass sugars by yeast. Using systems biology approaches, a tolerant industrial yeast strain against biomass pretreatment inhibitors was selected as a host in a genetic

engineering effort to improve its xylose utilization. An in vitro-synthesized yeast xylose isomerase gene (GenBank Accession No. JF261697) was engineered into a tolerant yeast by chromosomal integration as xylose utilization-driving route (Liu et al. 2011; Ma et al. unpublished data). Additional xylose transporter genes and downstream xylose utilization facilitating genes such as *XKS1* and *XYL2* were further introduced into the recombinant strains by genetic engineering. The resulted strain *S. cerevisiae* NRRL Y-50463 is able to grow and ferment ethanol on xylose as sole carbon source. It produced the highest ethanol yield to date for anaerobic cofermentation on mixed sugars of glucose and xylose by utilizing the xylose isomerase pathway (Table 1). The strain remains tolerant and able to in situ detoxify major inhibitory compounds derived from biomass pretreatment. Certain xylose transporter genes significantly improved xylose uptake in cells as measured by cellular accumulation of xylose (Moon et al. unpublished data). However, it is clear that without the functional yeast xylose isomerase, xylose transporter alone is unable to utilize xylose.

6 Molecular Mechanisms of Xylose Utilization

Molecular mechanisms of the improved xylose uptake and utilization are recently not clear. As *S. cerevisiae* is unable to sufficiently utilize the nonoxidative PPP (Gancedo and Lagunas 1973; Fiaux et al. 2003), enhancement of the PPP in xylose-utilizing strains by the overproduction of nonoxidative PPP enzymes has been attempted. For example, overexpression of the endogenous transaldolase gene (*TAL1*) (Walfridsson et al. 1995; Jin et al. 2005) and all four nonoxidative PPP genes, including transketolase (*TKL1*), ribulose-5-phosphate 3-epimerase (*RPE1*), and ribose-5-phosphate ketol-isomerase (*RKI1*) (Johansson and Hahn-Hägerdal 2002a, b; Karhumaa et al. 2005, 2007b; Kuyper et al. 2005a) in xylose-utilizing *S. cerevisiae* improved growth on xylose and the rate of xylulose consumption. To overcome the growth inhibition caused by overexpression of the *Sc*XK or *Ps*XK genes, overexpression of *TAL1* or deletion of the *PHO13* gene encoding alkaline phosphatase specific for *p*-nitrophenyl phosphate is a useful approach, as this enables growth on and fermentation of xylose (Ni et al. 2007; Van Vleet et al. 2008). Mutations of *S. cerevisiae* with improved xylose utilization showed enhanced expression of proteins involved in transport, initial xylose metabolism, and the PPP (Wahlbom et al. 2003b).

Data obtained by studies of genomics, transcriptomics, proteomics, metabolomics, and fluxomics are useful for targeting metabolic changes to enhance the rate and yield of ethanol production from xylose (Otero et al. 2007). Using these "omics" analyses, several groups have analyzed xylose-utilizing *S. cerevisiae* strains (Sedlak et al. 2003; Salusjärvi et al. 2003, 2006, 2008; Sonderegger et al. 2004; Jin et al. 2004; Bengtsson et al. 2008; Runquist et al. 2009b; Karhumaa et al. 2009). Global expression analyses of these metabolically engineered *S. cerevisiae* strains revealed that high levels of transcripts related to the tricarboxylic acid

(TCA) cycle and respiration were present during growth on xylose under oxygen-limited conditions. This finding suggests that xylose was used as a nonfermentable carbon source in xylose-utilizing *S. cerevisiae* and that respiratory proteins are induced in response to intracellular redox imbalances (Jin et al. 2004). Transcriptome analyses have also revealed that *S. cerevisiae* exhibits improved growth on xylose when *SOL3* and *TAL1* are upregulated, and *YLR042C*, *MNI1*, and *RPA49* are downregulated (Bengtsson et al. 2008). For recombinant *S. cerevisiae* strains using xylose reductase–xylitol dehydrogenase pathway, xylose was found not to be recognized as metabolic carbon source and starvation response was closely related by transcription analysis (Salusjärvi et al. 2006). Xylose was found only partially reprocessed for metabolic genes encoding proteins involved in respiration, TCA, glyoxylate cycle, and gluconeogenesis, and that xylose decreases the expression of several genes repressed by glucose via the SNF1/MIG1 pathway (Salusjärvi et al. 2008). Metabolic flux and genome-wide transcription analyses have verified that anaerobic growth on xylose causes upregulation of the oxidative PPP and gluconeogenesis (Runquist et al. 2009b) due to the necessity for $NADP^+$ reduction during anaerobic xylose metabolism (Jeppsson et al. 2002). Finally, proteomic analyses have identified 22 proteins in *S. cerevisiae* (e.g., ADH2, ALD4, ALD6, and GPP1) that exhibited increased expression during growth on xylose compared to growth on glucose (Salusjärvi et al. 2003) and have also demonstrated that the levels of ALD6, XR, XDH, and TKL1 were significantly elevated in a *S. cerevisiae* mutant with good xylose fermentation ability compared with the parental strain. Investigations on genome response using the yeast xylose isomerase–xylose transporter system for engineered *S. cerevisiae* are needed.

7 Conclusion and Perspectives

Balanced utilization of biomass sugars, particularly xylose, has become a focus of various research efforts for improvement of cellulosic ethanol production since last decade. Recombinant engineering of *S. cerevisiae* using heterologous genes such as XR, XDH, and XI generated a significant amount of knowledge and many strains that are capable of utilizing xylose at varied levels. The recent development of engineering synthesized yeast xylose isomerase, xylose transporters, and other xylose-utilizing genes into tolerant industrial yeast refreshing the effort. The successful outcome in applying systems biology marks a new phase in yeast strain development for improving biomass sugar utilizations for cellulosic ethanol production. As omics analyses of metabolically engineered strains are rapidly progressing, the practical application of more desirable strains capable of efficiently fermenting all biomass sugars, including xylose, found in lignocellulosic hydrolysates to ethanol may soon be realized on the commercial and industrial levels.

Acknowledgments The authors thank Drs. Shinichi Yano, Katsuji Murakami, Hiroyuki Inoue, Kenichiro Tsukahara, and Ohgiya Satoru (AIST), Keisuke Makino, Tsutomu Kodaki, Seiya Watanabe (Kyoto University), Takeshi Mizuno, Takafumi Yamashino (Nagoya University), and Mr. Osamu Takimura for helpful discussions. This study was supported in part by the New Energy and Industrial Technology Development Organization, Japan to AM and SS; and NIFA National Research Initiative Grant Award Project 2006-35504-17359 to ZLL.

References

Almeida JR, Hahn-Hägerdal B (2009) Developing *Saccharomyces cerevisiae* strains for second generation bioethanol: improving xylose fermentation and inhibitor tolerance. Int Sugar J 111:172–180

Amore R, Wilhelm M, Hollenberg CP (1989) The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. Appl Microbiol Biotechnol 30:351–357

Aristidou A, Penttilä M (2000) Metabolic engineering applications to renewable resource utilization. Curr Opin Biotechnol 11:187–198

Bengtsson O, Jeppsson M, Sonderegger M, Parachin NS, Sauer U, Hahn-Hägerdal B, Gorwa-Grauslund MF (2008) Identification of common traits in improved xylose-growing *Saccharomyces cerevisiae* for inverse metabolic engineering. Yeast 25:835–847

Brandberg T, Franzén CJ, Gustafsson L (2004) The fermentation performance of nine strains of *Saccharomyces cerevisiae* in batch and fed-batch cultures in dilute-acid wood hydrolysate. J Biosci Bioeng 98:122–125

Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:2304–2311

Bruinenberg PM, de Bot PHM, van Dijken JP, Scheffers WA (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. Appl Microbiol Biotechnol 18:287–292

Chang SF, Ho NW (1988) Cloning the yeast xylulokinase gene for the improvement of xylose fermentation. Appl Biochem Biotechnol 17:313–318

Chu BC, Lee H (2007) Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. Biotechnol Adv 25:425–441

Deng XX, Ho NWY (1990) Xylulokinase activity in various yeasts including *Saccharomyces cerevisiae* containing the cloned xylulokinase gene. Appl Biochem Biotechnol 24–25:193–199

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266

Does AL, Bisson LF (1989) Characterization of xylose uptake in the yeasts *Pichia heedii* and *S. stipitis*. Appl Environ Microbiol 55:159–164

Du J, Li S, Zhao H (2010) Discovery and characterization of novel d-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*. Mol Biosyst 6:2150–2156

Eliasson A, Boles E, Johansson B, Österberg M, Thevelein JM, Spencer-Martins I, Juhnke H, Hahn-Hägerdal B (2000a) Xylulose fermentation by mutant and wild-type strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:376–382

Eliasson A, Christensson C, Wahlbom CF, Hahn-Hägerdal B (2000b) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL1*, *XYL2*, and *XKS1* in mineral medium chemostat cultures. Appl Environ Microbiol 66:3381–3386

Eliasson A, Hofmeyr J-HS, Pedler S, Hahn-Hägerdal B (2001) The xylose reductase/xylitol dehydrogenase/xylulokinase ratio affects product formation in recombinant xylose-utilising *Saccharomyces cerevisiae*. Enzyme Microb Technol 29:288–297

Fiaux J, Cakar ZP, Sonderegger M, Wüthrich K, Szyperski T, Sauer U (2003) Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Eukaryot Cell 2:170–180

Gancedo JM, Lagunas R (1973) Contribution of the pentose phosphate pathway to glucose metabolism in *Saccharomyces cerevisiae*: a critical analysis on the use of labelled glucose. Plant Sci Lett 1:193–200

Gárdonyi M, Hahn-Hägerdal B (2003) The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. Enzyme Microb Technol 32:252–259

Gong CS, Cao NJ, Du J, Tsao GT (1999) Ethanol production from renewable resources. Adv Biochem Eng Biotechnol 65:207–241

Hahn-Hägerdal B, Wahlbom CF, Gárdonyi M, van Zyl WH, Cordero Otero RR, Jönsson LJ (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv Biochem Eng Biotechnol 73:53–84

Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007a) Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 74:937–953

Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF (2007b) Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 108:147–177

Hamacher T, Becker J, Gárdonyi M, Hahn-Hägerdal B, Boles E (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. Microbiology 148:2783–2788

Hector RE, Qureshi N, Hughes SR, Cotta MA (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. Appl Microbiol Biotechnol 80:675–684

Heer D, Sauer U (2008) Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. Microb Biotechnol 1:497–506

Ho NWY, Chen Z, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859

Ho NWY, Chen Z, Brainard AP, Sedlak M (1999) Successful design and development of genetically engineered *Saccharomyces* yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. Adv Biochem Eng Biotechnol 65:163–192

Hsiao HY, Chiang LC, Ueng PP, Tsao GT (1982) Sequential utilization of mixed monosaccharides by yeasts. Appl Environ Microbiol 43:840–845

Hughes SR, Sterner DE, Bischoff KM, Hector RE, Dowd PF, Qureshi N, Bang SS, Grynaviski N, Chakrabarty T, Johnson ET, Dien BS, Mertens JA, Caughey RJ, Liu S, Butt TR, LaBaer J, Cotta MA, Rich JO (2009) Engineered *Saccharomyces cerevisiae* strain for improved xylose utilization with a three-plasmid SUMO yeast expression system. Plasmid 61:22–38

Jeffries TW (2006) Engineering yeasts for xylose metabolism. Curr Opin Biotechnol 17:320–326

Jeffries TW, Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63:495–509

Jeffries TW, Shi NQ (1999) Genetic engineering for improved xylose fermentation by yeasts. Adv Biochem Eng Biotechnol 65:117–161

Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H, Jin YS, Passoth V, Richardson PM (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25:319–326

Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68:1604–1609

Jeppsson M, Träff K, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2003) Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. FEMS Yeast Res 3:167–175

Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal B, Gorwa-Grauslund MF (2006) The expression of a *Pichia stipitis* xylose reductase mutant with higher K_M for NADPH increases ethanol production from xylose recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 93:665–673

Jin YS, Jeffries TW (2003) Changing flux of xylose metabolites by altering expression of xylose reductase and xylitol dehydrogenase in recombinant *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 105–108:277–285

Jin YS, Ni H, Laplaza JM, Jeffries TW (2003) Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. Appl Environ Microbiol 69:495–503

Jin YS, Laplaza JM, Jeffries TW (2004) *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. Appl Environ Microbiol 70:6816–6825

Jin YS, Alper H, Yang YT, Stephanopoulos G (2005) Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through an inverse metabolic engineering approach. Appl Environ Microbiol 71:8249–8256

Johansson B, Hahn-Hägerdal B (2002a) The non-oxidative pentose phosphate pathway controls the fermentation rate of xylulose but not of xylose in *Saccharomyces cerevisiae* TMB3001. FEMS Yeast Res 2:277–282

Johansson B, Hahn-Hägerdal B (2002b) Overproduction of pentose phosphate pathway enzymes using a new CRE-loxP expression vector for repeated genomic integration in *Saccharomyces cerevisiae*. Yeast 19:225–231

Johansson B, Christensson C, Hobley T, Hahn-Hägerdal B (2001) Xylulokinase overexpression in two strains of *Saccharomyces cerevisiae* also expressing xylose reductase and xylitol dehydrogenase and its effect on fermentation of xylose and lignocellulosic hydrolysate. Appl Environ Microbiol 67:4249–4255

Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF (2005) Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22:359–368

Karhumaa K, Fromanger R, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007a) High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 73:1039–1046

Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007b) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 6:5

Karhumaa K, Påhlman AK, Hahn-Hägerdal B, Levander F, Gorwa-Grauslund MF (2009) Proteome analysis of the xylose-fermenting mutant yeast strain TMB 3400. Yeast 26:371–382

Katahira S, Ito M, Takema H, Fujita Y, Tanino T, Tanaka T, Fukuda H, Kondo A (2008) Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S. cerevisiae* via expression of glucose transporter Sut1. Enzyme Microb Technol 43:115–119

Kavanagh KL, Klimacek M, Nidetzky B, Wilson DK (2002) The structure of apo and holo forms of xylose reductase, a dimeric aldo-keto reductase from *Candida tenuis*. Biochemistry 41:8785–8795

Kavanagh KL, Klimacek M, Nidetzky B, Wilson DK (2003) Structure of xylose reductase bound to NAD^+ and the basis for single and dual co-substrate specificity in family 2 aldo-keto reductases. Biochem J 373:319–326

Kilian SG, van Uden N (1988) Transport of xylose and glucose in the xylose-fermenting yeast *Pichia stipitis*. Appl Microbiol Biotechnol 27:545–548

Kostrzynska M, Sopher CR, Lee H (1998) Mutational analysis of the role of the conserved lysine-270 in the *Pichia stipitis* xylose reductase. FEMS Microbiol Lett 159:107–112

Kötter P, Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776–783

Krahulec S, Klimacek M, Nidetzky B (2009) Engineering of a matched pair of xylose reductase and xylitol dehydrogenase for xylose fermentation by *Saccharomyces cerevisiae*. Biotechnol J 4:684–694

Krishnan MS, Ho NWY, Tsao GT (1999) Fermentation kinetics of ethanol production from glucose and xylose by recombinant *Saccharomyces* 1400(pLNH33). Appl Biochem Biotechnol 77–79:373–388

Kruckeberg AL (1996) The hexose transporter family of *Saccharomyces cerevisiae*. Arch Microbiol 166:283–292

Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP, Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4:69–78

Kuyper M, Winkler AA, van Dijken JP, Pronk JT (2004) Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res 4:655–664

Kuyper M, Hartog MM, Toirkens MJ, Almering MJ, Winkler AA, van Dijken JP, Pronk JT (2005a) Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res 5:399–409

Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT (2005b) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5:925–934

Leandro MJ, Gonçalves P, Spencer-Martins I (2006) Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose-H^+ symporter. Biochem J 395:543–549

Leandro MJ, Spencer-Martins I, Gonçalves P (2008) The expression in *Saccharomyces cerevisiae* of a glucose/xylose symporter from *Candida intermedia* is affected by the presence of a glucose/xylose facilitator. Microbiology 154:1646–1655

Leitgeb S, Petschacher B, Wilson DK, Nidetzky B (2005) Fine tuning of coenzyme specificity in family 2 aldo-keto reductases revealed by crystal structures of the Lys-274 → Arg mutant of *Candida tenuis* xylose reductase (AKR2B5) bound to NAD^+ and $NADP^+$. FEBS Lett 579:763–767

Ligthelm ME, Prior BA, du Preez JC, Bandt V (1988) An investigation of D-{1-^{13}C} xylose metabolism in *Pichia stipitis* under aerobic and anaerobic conditions. Appl Microbiol Biotechnol 28:293–296

Lin S, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642

Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31:345–352

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and 5-hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Liu ZL, Moon J, Andersh AJ, Slininger PJ, Weber S (2008a) Multiple gene mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and HMF by ethanologenic yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:743–753

Liu ZL, Saha BC, Slininger PJ (2008b) Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Wall JD, Harwood CS, Demain A (eds) Bioenergy. ASM Press, Washington DC

Liu ZL, Ma M, Moon J (2011) Newly engineering designed ethanologenic yeast *Saccharomyces cerevisiae* tolerates lignocellulose hydrolysates and utilizes heterogeneous biomass sugars for cellulosic ethanol conversion. Abstract. American Society for Microbiology 111th Annual Meeting.

Lönn A, Gárdonyi M, van Zyl W, Hahn-Hägerdal B, Otero RC (2002) Cold adaptation of xylose isomerase from *Thermus thermophilus* through random PCR mutagenesis. Gene cloning and protein characterization. Eur J Biochem 269:157–163

Lönn A, Träff-Bjerre KL, Cordero Otero RR, van Zyl WH, Hahn-Hägerdal B (2003) Xylose isomerase activity influences xylose fermentation with recombinant *Saccharomyces cerevisiae* strains expressing mutated *xylA* from *Thermus thermophilus*. Enzyme Microb Technol 32:567–573

Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009a) Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl Microbiol Biotechnol 82:1067–1078

Madhavan A, Tamalampudi S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009b) Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization. Appl Microbiol Biotechnol 82:1037–1047

Martín C, Jönsson LJ (2003) Comparison of the resistance of industrial and laboratory strains of *Saccharomyces cerevisiae* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. Enzyme Microb Technol 32:386–395

Martín C, Marcet M, Almazán O, Jönsson LJ (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767–1773

Matsushika A, Sawayama S (2008) Efficient bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* requires high activity of xylose reductase and moderate xylulokinase activity. J Biosci Bioeng 106:306–309

Matsushika A, Sawayama S (2010) Effect of initial cell concentration on ethanol production by flocculent *Saccharomyces cerevisiae* with xylose-fermenting ability. Appl Biochem Biotechnol 162:1952–1960

Matsushika A, Watanabe S, Kodaki T, Makino K, Sawayama S (2008a) Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, $NADP^+$-dependent xylitol dehydrogenase, and xylulokinase. J Biosci Bioeng 105:296–299

Matsushika A, Watanabe S, Kodaki T, Makino K, Inoue H, Murakami K, Takimura O, Sawayama S (2008b) Expression of protein engineered $NADP^+$-dependent xylitol dehydrogenase increase ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:243–255

Matsushika A, Inoue H, Murakami K, Takimura O, Sawayama S (2009a) Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Bioresour Technol 100:2392–2398

Matsushika A, Inoue H, Watanabe S, Kodaki T, Makino K, Sawayama S (2009b) Efficient bioethanol production by recombinant flocculent *Saccharomyces cerevisiae* with genome-integrated $NADP^+$-dependent xylitol dehydrogenase gene. Appl Environ Microbiol 75:3818–3822

Matsushika A, Inoue H, Kodaki T, Sawayama S (2009c) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol 84:37–53

Matsushika A, Oguri E, Sawayama S (2010) Evolutionary adaptation of recombinant shochu yeast for improved xylose utilization. J Biosci Bioeng 110:102–105

Moes CJ, Pretorius IS, van Zyl WH (1996) Cloning and expression of the *Clostridium thermosulfurogenes* D-xylose isomerase gene (*xylA*) in *Saccharomyces cerevisiae*. Biotechnol Lett 18:269–274

Ni H, Laplaza M, Jeffries TW (2007) Transposon mutagenesis to improve the growth of recombinant *Saccharomyces cerevisiae* on D-xylose. Appl Environ Microbiol 73:2061–2066

Olsson L, Hahn-Hägerdal B (1993) Fermentative performance of bacteria and yeast in lignocellulose hydrolysates. Process Biochem 28:249–257

Olsson L, Nielsen J (2000) The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: utilization of industrial media. Enzyme Microb Technol 26:785–792

Otero JM, Panagiotou G, Olsson L (2007) Fueling industrial biotechnology growth with bioethanol. Adv Biochem Eng Biotechnol 108:1–40

Petschacher B, Nidetzky B (2005) Engineering *Candida tenuis* xylose reductase for improved utilization of NADH: antagonistic effects of multiple side chain replacements and performance of site-directed mutants under simulated in vivo conditions. Appl Environ Microbiol 71:6390–6393

Petschacher B, Nidetzky B (2008) Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 7:9

Petschacher B, Leitgeb S, Kavanagh KL, Wilson DK, Nidetzky B (2005) The coenzyme specificity of *Candida tenuis* xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. Biochem J 385:75–83

Pitkänen JP, Rintala E, Aristidou A, Ruohonen L, Penttilä M (2005) Xylose chemostat isolates of *Saccharomyces cerevisiae* show altered metabolite and enzyme levels compared with xylose, glucose, and ethanol metabolism of the original strain. Appl Microbiol Biotechnol 67:827–837

Rodriguez-Pena JM, Cid VJ, Arroyo J, Nombela C (1998) The YGR194c (XKS1) gene encodes the xylulokinase from the budding yeast *Saccharomyces cerevisiae*. FEMS Microbiol Lett 162:155–160

Runquist D, Fonseca C, Rådström P, Spencer-Martins I, Hahn-Hägerdal B (2009a) Expression of the Gxf1 transporter from *Candida intermedia* improves fermentation performance in recombinant xylose-utilizing *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 82:123–130

Runquist D, Hahn-Hägerdal B, Bettiga M (2009b) Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. Microb Cell Fact 8:49

Runquist D, Hahn-Hägerdal B, Rådström P (2010) Comparison of heterologous xylose transporters in recombinant Saccharomyces cerevisiae. Biotechnol Biofuels 3:5

Saier MH Jr, Tran CV, Barabote RD (2006) TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic Acids Res 34(Database issue): D181–D186

Saloheimo A, Rauta J, Stasyk OV, Sibirny AA, Penttilä M, Ruohonen L (2007) Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. Appl Microbiol Biotechnol 74:1041–1052

Salusjärvi L, Poutanen M, Pitkänen JP, Koivistoinen H, Aristidou A, Kalkkinen N, Ruohonen L, Penttilä M (2003) Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*. Yeast 20:295–314

Salusjärvi L, Pitkänen JP, Aristidou A, Ruohonen L, Penttilä M (2006) Transcription analysis of recombinant *Saccharomyces cerevisiae* reveals novel responses to xylose. Appl Biochem Biotechnol 128:237–261

Salusjärvi L, Kankainen M, Soliymani R, Pitkänen JP, Penttilä M, Ruohonen L (2008) Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 7:18

Sarthy AV, McConaughy BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD (1987) Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. Appl Environ Microbiol 53:1996–2000

Sauer U (2001) Evolutionary engineering of industrially important microbial phenotypes. Adv Biochem Eng Biotechnol 73:129–169

Sedlak M, Ho NWY (2004) Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. Yeast 21:671–684

Sedlak M, Edenberg HJ, Ho NWY (2003) DNA microarray analysis of the expression of the genes encoding the major enzymes in ethanol production during glucose and xylose co-fermentation by metabolically engineered *Saccharomyces* yeast. Enzyme Microb Technol 33:19–28

Slininger PJ, Bolen PL, Kurtzman CP (1987) *Pachysolen tannophilus*: properties and process considerations for ethanol production from D-xylose. Enzyme Microb Technol 9:5–15

Sonderegger M, Sauer U (2003) Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol 69:1990–1998

Sonderegger M, Jeppsson M, Hahn-Hägerdal B, Sauer U (2004) Molecular basis for anaerobic growth of *Saccharomyces cerevisiae* on xylose, investigated by global gene expression and metabolic flux analysis. Appl Environ Microbiol 70:2307–2317

Souto-Maior AM, Runquist D, Hahn-Hägerdal B (2009) Crabtree-negative characteristics of recombinant xylose-utilizing *Saccharomyces cerevisiae*. J Biotechnol 143:119–123

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11

Tantirungkij M, Nakashima N, Seki T, Yoshida T (1993) Construction of xylose-assimilating *Saccharomyces cerevisiae*. J Ferment Bioeng 75:83–88

Taylor M, Tuffin M, Burton S, Eley K, Cwan D (2008) Microbial responses to solvent and alcohol stress. Biotechnol J 3:1388–1397

Toivari MH, Aristidou A, Ruohonen L, Penttilä M (2001) Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (*XKS1*) and oxygen availability. Metab Eng 3:236–249

Träff KL, Otero Cordero RR, van Zyl WH, Hahn-Hägerdal B (2001) Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xylA* and *XKS1* genes. Appl Environ Microbiol 67:5668–5674

Träff-Bjerre KL, Jeppsson M, Hahn-Hägerdal B, Gorwa-Grauslund MF (2004) Endogenous NADPH-dependent aldose reductase activity influences product formation during xylose consumption in recombinant *Saccharomyces cerevisiae*. Yeast 21:141–150

van Maris AJ, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MA, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Leeuwenhoek 90:391–418

van Maris AJ, Winkler AA, Kuyper M, de Laat WT, van Dijken JP, Pronk JT (2007) Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. Adv Biochem Eng Biotechnol 108:179–204

Van Vleet JH, Jeffries TW (2009) Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 20:300–306

Van Vleet JH, Jeffries TW, Olsson L (2008) Deleting the *para*-nitrophenyl phosphatase (pNPPase), *PHO13*, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose. Matab Eng 10:360–369

van Zyl C, Prior BA, Kilian SG, Brandt EV (1993) Role of D-ribose as a cometabolite in D-xylose metabolism by *Saccharomyces cerevisiae*. Appl Environ Microbiol 59:1487–1494

Verho R, Richard P, Jonson PH, Sundqvist L, Londesborough J, Penttilä M (2002) Identification of the first fungal NADP-GAPDH from *Kluyveromyces lactis*. Biochemistry 41:13833–13838

Verho R, Londesborough J, Penttilä M, Richard P (2003) Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:5892–5897

Wahlbom CF, van Zyl WH, Jönsson LJ, Hahn-Hägerdal B, Cordero Otero RR (2003a) Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res 3:319–326

Wahlbom CF, Cordero Otero RR, van Zyl WH, Hahn-Hägerdal B, Jönsson LJ (2003b) Molecular analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. Appl Environ Microbiol 69:740–746

Walfridsson M, Hallborn J, Penttilä M, Keränen S, Hahn-Hägerdal B (1995) Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the *TKL1* and *TAL1* genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. Appl Environ Microbiol 61:4184–4190

Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B (1996) Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus*

xylA gene, which expresses an active xylose (glucose) isomerase. Appl Environ Microbiol 62:4648–4651

Walfridsson M, Anderlund M, Bao X, Hahn-Hägerdal B (1997) Expression of different levels of enzymes from the *Pichia stipitis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. Appl Microbiol Biotechnol 48:218–224

Wang PY, Schneider H (1980) Growth of yeasts on D-xylulose. Can J Microbiol 26:1165–1168

Watanabe S, Kodaki T, Makino K (2005) Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. J Biol Chem 280:10340–10349

Watanabe S, Pack SP, Saleh AA, Annaluru N, Kodaki T, Makino K (2007a) Positive effect of the decreased NADPH-preferring activity of xylose reductase from *Pichia stipitis* on ethanol production using xylose-fermenting recombinant *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 71:1365–1369

Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K (2007b) Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein engineered NADH-preferring xylose reductase from *Pichia stipitis*. Microbiology 153:3044–3054

Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K (2007c) Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein engineered $NADP^+$-dependent xylitol dehydrogenase. J Biotechnol 130:316–319

Weierstall T, Hollenberg CP, Boles E (1999) Cloning and characterization of three genes (*SUT1-3*) encoding glucose transporters of the yeast *Pichia stipitis*. Mol Microbiol 31:871–883

Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJ (2009) A novel evolutionary engineering approach for accelerated utilization of glucose, xylose and arabinose mixtures by engineered *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:907–914

Yamanaka K (1969) Inhibition of D-xylose isomerase by pentitols and D-lyxose. Arch Biochem Biophys 131:502–506

Yu S, Jeppsson H, Hahn-Hägerdal B (1995) Xylulose fermentation by *Saccharomyces cerevisiae* and xylose-fermenting yeast strains. Appl Microbiol Biotechnol 44:314–320

Zaldivar J, Borges A, Johansson B, Smits HP, Villas-Bôas SB, Nielsen J, Olsson L (2002) Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 59:436–442

Zhong C, Lau MW, Balan V, Dale BE, Yuan YJ (2009) Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl Microbiol Biotechnol 84:667–676

Genomics on Pretreatment Inhibitor Tolerance of *Zymomonas mobilis*

Shihui Yang, Martin Keller, and Steven D. Brown

Contents

1 Introduction 162
2 Genome Annotation of ZM4 Using Systems Biology Studies 163
3 Identification of Genes Tolerant to Acetate 164
3.1 *nhaA* 164
3.2 *hfq* 169
3.3 *nhaA* and *hfq* 170
3.4 *himA* 170
4 Heterologous Expression for Strain Improvement 171
5 Conclusion and Perspectives 171
References 172

Abstract The development and use of robust ethanologenic microorganisms resistant to industrially relevant pretreatment inhibitors will be a critical component in the successful generation of biofuel on the industrial scale. Recent progress to understand the genetic basis of pretreatment inhibitor tolerance using genomics and systems biology tools for metabolic engineering for the model ethanologenic bacterium *Zymomonas mobilis* is reviewed in this chapter. The importance of accurate genome annotations and the integration of systems biology data for annotation improvement are highlighted, and case studies that describe the identification and characterization of the *Z. mobilis nhaA*, *hfq*, and *himA* inhibitor tolerance related gene targets are presented.

S. Yang • M. Keller • S.D. Brown (✉)
Biosciences Division and BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
e-mail: brownsd@ornl.gov

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_7,

1 Introduction

A core challenge for next-generation biomass-based cellulosic biofuels is overcoming biomass recalcitrance, or gaining access to its sugars that can then be converted to biofuels (Himmel et al. 2007; Alper and Stephanopoulos 2009). Biomass pretreatment is necessary for optimal release of C-5 and C-6 sugars but can also create a range of inhibitory by-products such as aldehydes, ketones, organic acids, and phenols (Pienkos and Zhang 2010; Palmqvist and Hahn-Hägerdal 2000; Klinke et al. 2004; Liu and Blaschek 2010). Synergistic or additive inhibitory effects are also likely among different hydrolysate inhibitors or metabolic by-products generated during the fermentation such as ethanol, acetate, and lactate (see recent reviews (Mills et al. 2009; Almeida et al. 2007)). An increased lag phase and slower growth increase the biofuel production costs due to reduced production rates and decreased yields (Kadar et al. 2007; Takahashi et al. 1999).

Acetic acid is one major organic acid inhibitor. It is generated by the de-acetylation of hemicelluloses during the pretreatment of biomass. At pH 5.0, about 36% of acetic acid is in the uncharged and undissociated form (HAc). In this form, it is able to pass through the bacterial plasma membrane, leading to uncoupling of the HAc and anion accumulation which causes cytoplasmic acidification (Lawford and Rousseau 1993). Its importance comes from the significant concentrations of acetate that are produced relative to fermentable sugars (McMillan 1994). The produced acetate concentration is also dependent on the feedstock used during the conversion process. An approach to overcoming possible inhibition caused by pretreatment is to remove the inhibitors from the biomass physically or chemically after pretreatment (Pienkos and Zhang 2010). This requires additional equipment and time, thus leading to higher cost. For example, acetate removal processes have been described, but they are energy- or chemical-intensive, and a full-cost analysis has not been reported (McMillan 1994). Applications of inhibitor-tolerant microorganisms appear promising for lower-cost cellulosic biofuel conversion (Almeida et al. 2007; Liu and Blaschek 2010; Liu et al. 2004, 2005, 2008, 2009; Liu and Moon 2009).

Yeast strains are among the current leading industrial biocatalyst microorganisms for fuel production (Hahn-Hagerdal et al. 2006). However, bacteria such as *Escherichia coli, Zymomonas mobilis*, and others are being engineered, developed, and deployed to address commercially important inoculum requirements (Dien et al. 2003; Alper and Stephanopoulos 2009). *Z. mobilis* is a Gram-negative facultative anaerobic bacterium with desirable industrial biocatalyst characteristics, such as high specific productivity, high ethanol yield, and ethanol tolerance (12% v/v) (Dien et al. 2003; Panesar et al. 2006; Rogers et al. 2007). The genome sequence of strain ZM4 has been determined (Seo et al. 2005) and an updated annotation was released recently (Yang et al. 2009a). In addition, the genome sequence and annotation of *Z. mobilis* NCIMB 11163 strain has been reported (Kouvelis et al. 2009) with more strains to be finished or sequenced. Wild-type *Z. mobilis* strains can only utilize a limited range of carbon sources, namely, glucose, fructose, and sucrose. To overcome this limitation, recombinant strains have been engineered to ferment hexose and pentose sugars such

as xylose, arabinose, and other substrates with high yields (Deanda et al. 1996; Zhang et al. 1995), but a low tolerance to acetic acid and a decreased tolerance to ethanol have been reported (Dien et al. 2003; Lawford and Rousseau 1998; Lawford et al. 2001; Ranatunga et al. 1997). In addition, recent achievements to improve transformation efficiency by modifying the DNA restriction-modification systems (Kerr et al. 2010), cellulase expression and secretion (Linger et al. 2010), as well as the genome-scale modeling and *in silico* analysis (Widiastuti et al. 2010), will aid future metabolic engineering and synthetic biology endeavors greatly.

The development and use of robust ethanol-generating microorganisms resistant to industrially relevant inhibitors and with a high-yield ethanol production will be a critical component in the successful generation of fuel ethanol on the industrial scale. However, limited progress has been made in understanding the genetic basis of inhibitor tolerance (Stephanopoulos 2007), and there are few examples of metabolic engineering with systems biology tools for bioprocess development to date (Park et al. 2008). In this chapter, the focus is on genome-based approaches to elucidate molecular mechanisms of inhibitor tolerance for *Z. mobilis*.

2 Genome Annotation of ZM4 Using Systems Biology Studies

Genome sequencing projects provide opportunities for fundamental insights and facilitate strain development (Jeffries 2005). The next generation of new sequencing technologies are delivering fast and relatively inexpensive genome information (see recent reviews (MacLean et al. 2009; Metzker 2010)). Since the first complete microbial genome was published in July 1995 (Fleischmann et al. 1995), the number of finished microbial genomes has grown rapidly. As of August 24, 2010, 1,213 microbial genome sequencing projects have been completed with 3,422 in progress. Detailed information on prokaryotic genome sequencing projects can be accessed at the NCBI Microbial Genomes Resources database: http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_growth.html or the Genomes OnLine Database at: http://www.genomesonline.org/.

The majority of annotation efforts have focused on automatic bioinformatics approaches that are indispensable and based on similarity searches. However, there are issues related to the quality of genome sequencing, and intrinsic annotation errors have also been raised (Devos and Valencia 2001). Inaccurate prediction of open reading frames (ORFs), hypothetical protein descriptions, and discovery of new regulatory elements such as small regulatory are just some of the examples of issues related to genome sequences. On occasions, scientists are faced with different annotation versions generated by different groups for the same genome sequence.

In the case of *Z. mobilis* ZM4, many differences can be seen between the primary annotation and one performed by the J. Craig Venter Institute (JCVI) (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntzm01). Differential gene expression for ORFs predicted by JCVI but absent from the primary annotation has been reported

(Yang et al. 2009b). In addition, the existence of ZM4 plasmids has been reported previously (Yablonsky et al. 1988), but they were not included in the original genome annotation for the strain (Seo et al. 2005). The ZM4 genome annotation has been improved using an updated microbial genome annotation pipeline, the addition of annotated DNA sequences for five plasmids, and data generated from several proteomics studies (Yang et al. 2009a). Almost one-third of the original genome ORF predictions were changed, including important genes such as *nhaA* (ZMO0119) (see detailed descriptions at a later section). The 156 new plasmid gene models represent coding sequences for important genes like an iron-containing alcohol dehydrogenase, hypothetical genes with unknown functions, genes for plasmid maintenance, transport, regulation, metabolism, as well as genes belonging to restriction-modification systems and phage-related genes (Yang et al. 2009a). It is therefore feasible to apply proteomics and next-generation sequencing information for genome annotation improvements, an activity that has received extensive attention recently with several other genome annotations undergoing similar improvements (Armengaud 2009; Baudet et al. 2010; Payne et al. 2010; Wright et al. 2009). The accurate *Z. mobilis* ZM4 genome sequence and annotation are essential components for successful systems biology studies in this and other important ethanologenic microorganisms. In the case of the *Z. mobilis* ZM4 genome update, the improvement was conducted in collaboration with the authors of the primary sequence, which meant the research community was better served by a unified GenBank accession number.

3 Identification of Genes Tolerant to Acetate

Classic strain development that combines random mutagenesis and selection has a long history of success in generation of biocatalysts with industrially designed traits (Parekh et al. 2000; Patnaik 2008). However, the genetic loci contributing to the phenotypic strain changes can be difficult to identify. Systems biology tools and greater access to next-generation sequencing technologies are being increasingly exploited to gain insights into molecular mechanisms that link genotypes to important phenotypes. This section discusses strategies of tolerant gene identifications against acetate in *Z. mobilis*.

3.1 nhaA

An acetate-tolerant *Z. mobilis* mutant (AcR) was created via chemical mutagenesis with *N*-methyl *N*′-nitro *N*-nitrosoguanidine and selection in a continuous culture with a progressively increasing concentration of sodium acetate in the medium feed (Joachimstahl et al. 1998). AcR can efficiently produce ethanol in the presence of 20 g/L NaAc, while the parent organism ZM4 is inhibited above 12 g/L NaAc under

the same conditions (Joachimstahl et al. 1998). Acetic acid was inhibitory to the wild-type-derived strain ZM4(pZB5), which contains the plasmid pZB5 expressing *Escherichia coli* genes for pentose metabolism and xylose assimilation (Zhang et al. 1995) on xylose medium. The major inhibition mechanisms were possibly the intracellular de-energization and acidification (Kim et al. 2000). A recombinant strain was generated by transforming plasmid pZB5 into the AcR background, which can utilize both xylose and glucose with increased acetate resistance and improved fermentation characteristics in the presence of 12 g/L NaAc (Jeon et al. 2002). However, strain AcR was generated while many systems biology tools were being developed or had yet to be conceived, and the molecular mechanism of AcR sodium acetate tolerance was elusive until recently (Yang et al. 2010a).

The mutations in the AcR strain were identified and confirmed through the combination of microarray-based comparative genome sequencing (CGS), next-generation 454-pyroresequencing, and Sanger sequencing (Fig. 1) (Yang et al. 2010a). The CGS results from AcR identified a 1,461-bp (~1.5 kb) region of deleted DNA, which was confirmed using polymerase chain reaction, agarose gel electrophoresis, and Sanger sequencing analysis (Fig. 2). CGS results also identified 38

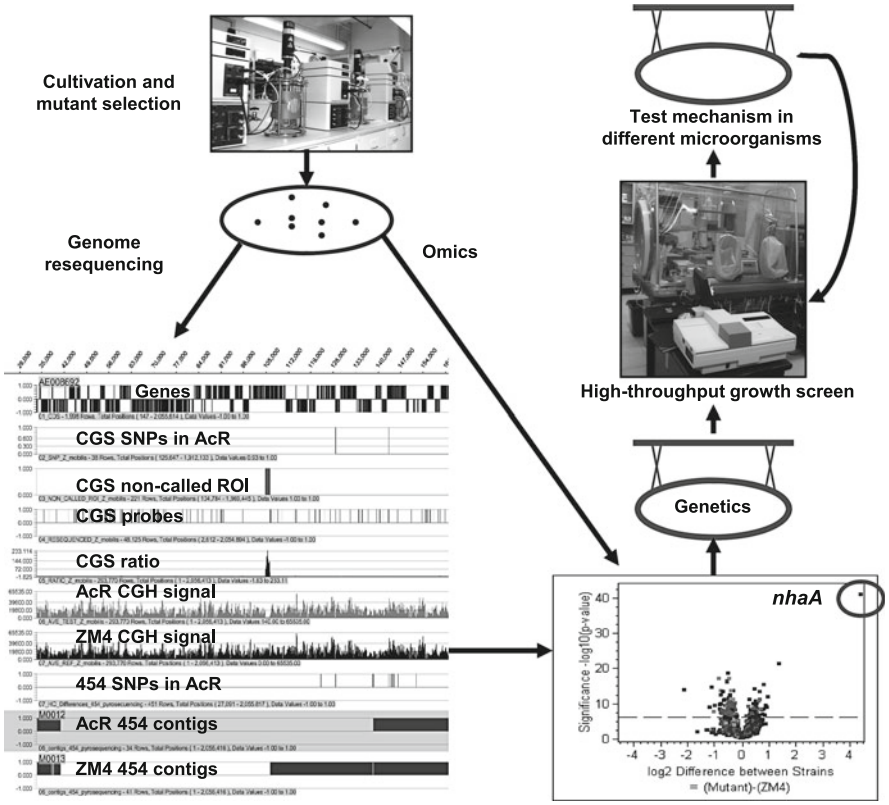

Fig. 1 Overview of the scheme used to identify sodium proton antiporter tolerance mechanisms

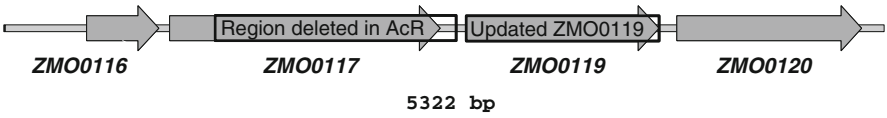

Fig. 2 *Z. mobilis nhaA* (ZMO0119) and its adjacent genes. *ZMO0116*, *ZMO0117*, *ZMO0119*, and *ZMO0120* indicate *Z. mobilis* ZM4 genes. The open box labeled "Region deleted in AcR" is present in ZM4 but deleted in the AcR mutant. The open box labeled "Updated ZMO0119" represents the updated annotation of ZMO0119, which was also used for *nhaA* overexpression plasmid p42-0119 construction

putative AcR single-nucleotide polymorphisms (SNPs), 26 of which were within coding regions and 12 within intergenic regions. From the 454-pyrosequencing shotgun and paired-end sequencing reads generated for ZM4 and AcR, 200 and 219 high confidence differences (HCDiffs) were identified for strains ZM4 and AcR, respectively, compared to the ZM4 reference genome (GenBank accession: AE008692) (Seo et al. 2005). An analysis of the putative mutations shared between ZM4 and AcR identified that most did not contribute to the AcR phenotype and led to improvements in the ZM4 chromosome sequence (Yang et al. 2009a). Only two confirmed SNPs were unique to AcR, with one synonymous SNP (i.e., no change at the amino acid level) found within *ZMO1184* encoding a hypothetical protein and a nonsynonymous SNP in *kup* (*ZMO1209*) encoding a putative potassium transporter. Therefore, the only differences between strains AcR and ZM4 after the ZM4 reference genome annotation update were the 1.5-kb deletion region that truncated *ZMO0117* and DNA upstream of the *nhaA* gene *ZMO0119* (Fig. 2), and two SNPs that affected *ZMO1184* and *ZMO1209*.

To further investigate the correlation between genotypic differences with phenotypic changes, transcriptomics studies were conducted to compare gene expression profiles between wild-type ZM4 and the acetate-tolerant mutant AcR under selective conditions. An analysis of variance (ANOVA) was conducted using JMP Genomics (SAS Institute Inc., Cary, NC) to identify significant differences in exponential and stationary phase transcriptomic profiles for ZM4 and AcR growing either in the presence of NaCl (146 mM or 8.6 g/L NaCl, pH 5.0) or NaAc (146 mM or 12 g/L NaAc, pH 5.0) (Fig. 1). Microarray analysis showed that *nhaA* expression was significantly increased (>16-fold) in strain AcR compared to ZM4 under all conditions tested (Fig. 1). The 1,461-bp deletion of AcR included a 1,363-bp region of *ZMO0117* with only a 275-bp 5′ fragment left and a 160-bp ZM4 *nhaA* upstream region with only 98-bp of the *nhaA* upstream region unchanged in AcR (Fig. 2). A consistently decreased *ZMO0117* signal was detected in each condition in the AcR strain compared to that of ZM4 in transcriptomics studies (Fig. 1). These findings suggested that the deletion in AcR enhanced the *nhaA* expression and likely led to enhanced NaAc tolerance in strain AcR.

To test the hypothesis that the deletion in AcR resulted in higher *nhaA* expression, which augmented NaAc tolerance, a deletion mutant ZM4DM0117 was generated to mimic the AcR 1,461-bp deletion in the wild-type ZM4 strain background by marker exchange (Fig. 2). To test the influence of *ZMO0117* on NaAc tolerance, a *ZMO0117*

insertion mutant strain ZM4IM0117 was constructed (Yang et al. 2010a). The *ZMO0118* gene was combined with *ZMO0119* in the recent update to the ZM4 genome (Yang et al. 2009a, shown in Fig. 2), which demonstrates the importance of working with the best available genome annotation.

To test the correlation between *nhaA* overexpression and NaAc tolerance, a plasmid p42-0119 for *nhaA* overexpression was generated and introduced into wild-type ZM4 background through conjugation and selection (Yang et al. 2010a). The overexpression and mutant strains grew similarly to wild-type ZM4 under anaerobic conditions in RM broth without NaAc supplementation (Fig. 3a). ZM4 wild type and the ZM4IM0117 were unable to grow with the supplementation of 195 mM (or 16 g/L) NaAc at pH 5.0, while the positive control strain AcR grew well (Fig. 3b). The expression of *nhaA* in ZM4 via plasmid p42-0119 restored the growth of ZM4 under these selective conditions, reaching three-fourths of the AcR growth rate. The final cell density (OD_{600nm}) of ZM4 (p42-0119) was only 13% less than that of AcR. ZM4DM0117 was able to grow in the presence of NaAc, achieving more than half of the growth rate and three-fourths of the final cell density of the AcR strain. The similar growth for the insertional mutant ZM4IM0117 as wild-type ZM4 indicated ZM0117 was not responsible for NaAc tolerance. ZM4 NaAc tolerance was augmented substantively by either additional *nhaA* copies provided via plasmid p42-0119 or by recreating the deleted DNA region of AcR in ZM4 wild-type background, which further suggested that the deletion in AcR truncated the *nhaA* promoter region resulted in higher *nhaA* expression, and in turn conferred the tolerance against NaAc.

To investigate the role of *nhaA* with different forms of acetate, ZM4 and AcR strains were grown with the supplementation of the same molar concentrations (195 mM) of sodium chloride (NaCl), NaAc, potassium acetate (KAc), or ammonium acetate (NH_4OAc) (Yang et al. 2010a). Both the sodium and acetate ions had an inhibitory effect on the growth of both *Z. mobilis* wild-type ZM4 and AcR, with decreases in both growth rate and final cell density. The acetate ion was more toxic than the sodium ion. *Z. mobilis* grew more rapidly in the presence of 195 mM NaCl, and the final cell density was higher compared to growth with the supplementation of same molar concentration of NH_4OAc or KAc. At the same molar concentration (195 mM), NaAc was more toxic than KAc or NH_4OAc for ZM4, and the combination of elevated Na^+ and Ac^- ions exerted a synergistic inhibitory effect on ZM4, with its growth totally inhibited.

The AcR strain was selected for sodium acetate tolerance (Joachimstahl et al. 1998). It also has an enhanced tolerance to NaCl, but not NH_4OAc or KAc as compared to the *Z. mobilis* wild-type ZM4 (Yang et al. 2010a). Strain ZM4DM0117 and ZM4 harboring the *nhaA* expression plasmid p42-0119 similarly had enhanced tolerance to NaCl that did not extend to NH_4OAc or KAc. The increased tolerance to NaAc for these strains therefore may be due mostly to an increased tolerance to the sodium ion arising from overexpression of the Na^+/H^+ antiporter gene *nhaA*. The strains were also tested for tolerance to other pretreatment inhibitors such as furfural, HMF, or vanillin, and advantages were not observed. These data again further suggested that NhaA mostly confers enhanced specific tolerance to Na^+ but not to

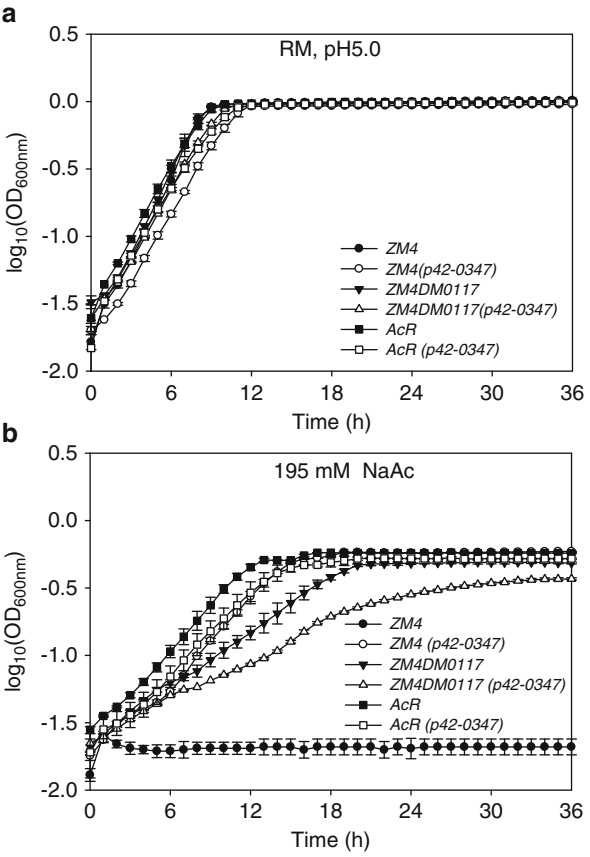

Fig. 3 Higher levels of *Z. mobilis* NaAc tolerance were not achieved through overexpression of both *hfq* and *nhaA*. The impact of Hfq overexpression (via p42-0347) in *Z. mobilis* wild type, acetate-tolerant mutant AcR, and a *Z. mobilis* deletion mutant ZM4DM0117 was assessed at different concentrations of NaAc and compared to that of corresponding parental strains: (**a**) RM broth only without NaAc supplementation as control and (**b**) RM broth with 195 mM NaAc). The growth of *Z. mobilis* strains were monitored by Bioscreen C (Growth Curves USA, NJ) under anaerobic conditions. Strains included in this study were: ZM4 (*Z. mobilis* ZM4 wild-type), AcR (previously described ZM4-derived acetate-tolerant mutant), AcR(p42-0347) (AcR containing a gateway plasmid p42-0347 for *hfq* (ZMO0347) expression), ZM4(p42-0347) (ZM4 containing a gateway plasmid p42-0347 for *hfq* (ZMO0347) expression), ZM4DM0117 (a deletion mutant of ZM4 that mimics the 1.5-Kb deletion in AcR), and ZM4DM0117(p42-0347) (ZM4DM0117 containing a gateway plasmid p42-0347 for *hfq* (ZMO0347) expression). This experiment has been repeated at least three times with similar results. Duplicates were used for each condition

other inhibitors, which reinforces the idea that "you get what you select for." Therefore, inhibitors used in selection regimes need to reflect the real conditions for desired performance where strains are also likely to face a number of different inhibitors.

A similar approach could be used for two other random mutants in the American Type Culture Collection (ATCC), the *Z. mobilis* (ATCC 31822) flocculent mutant

strain ZM401 and the ethanol-tolerant *Z. mobilis* mutant ZM481 (ATCC 31823). The investigation of the genetic differences between the wild-type and mutant strains may provide molecular mechanisms for ethanol tolerance and enhanced flocculation for strain development purposes. The development of high-throughput random mutant generation and selection for improved industrial processing traits, such as those tolerant to high substrate loading and a high concentration of hydrolysate, is needed to further develop industrial microorganisms, and subsequent characterization will allow for more-rapid strain development.

3.2 hfq

Z. mobilis ZM4 gene expression and metabolomic profiles during aerobic and anaerobic conditions were investigated, and it was determined that the ethanol production by *Z. mobilis* decreased with several inhibitory secondary metabolites produced in aerobic conditions (Yang et al. 2009b). This study also revealed that the expression of the putative *hfq* gene ZMO0347 was increased in anaerobic stationary phase compared to that in aerobic conditions (Yang et al. 2009b). Hfq is an RNA chaperone with pleiotropic regulatory roles involved in numerous stress responses (Tsui et al. 1994; Sittka et al. 2008; Zhang et al. 2003; Valentin-Hansen et al. 2004). However, little was known about the role of *Z. mobilis* Hfq in multiple pretreatment inhibitor tolerances until a recent reverse genetics study (Yang et al. 2010b). In this study, an *hfq* insertional mutant was generated in an ZM4 acetate-tolerant strain AcR with the pKnock-Km suicide plasmid system, and plasmid p42-0347 overexpressing *hfq* gene ZMO0347 was introduced into ZM4 wild type, acetate-tolerant mutant AcR, and *hfq* mutant AcRIM0347 by conjugation and selection (Yang et al. 2010b).

An *hfq* mutant (strain AcRIM0347) was unable to grow with the supplementation of 195 mM ammonium acetate or potassium acetate (Yang et al. 2010b). Both the final cell density and the growth rate of the *hfq* mutant were reduced by at least 25% and about 60% in the presence of 195 mM sodium chloride or sodium acetate as compared to that of the parental strain AcR. Consistent with previous reports (Joachimstahl et al. 1998; Yang et al. 2010a), the growth of wild-type ZM4 was completely inhibited in the presence of 195 mM sodium acetate. The introduction of an *hfq*-expressing plasmid (p42-0347) into wild-type ZM4 allowed wild-type ZM4 to obtain a similar growth rate and final cell density to those of acetate-tolerant strain AcR with the supplementation of 195 mM sodium acetate (Yang et al. 2010b). As *hfq* plays a central role in normal *Z. mobilis* physiology, the growth rate of *hfq* mutant AcRIM0347 was reduced to about 20% even without any inhibitor in rich medium (RM) although the final cell density of AcRIM0347 was similar to that of the AcR parental strain. The resistance of AcR to both sodium ion and acetate ion decreased when the *hfq* gene of AcR was inactivated by an insertional mutation. The AcRIM0347 *hfq* mutation was complemented partially by the introduction of an *hfq*-expressing plasmid p42-0347 into the strain.

The reduced inhibitor tolerance of an *hfq* mutant of acetate-tolerant strain AcR and enhanced acetate tolerance of the acetate-sensitive *Z. mobilis* wild-type strain by *hfq* overexpression indicated that *hfq* is important for optimal *Z. mobilis* growth. In addition, the study also showed the possibility to identify inhibitor-tolerant gene targets by top-down systems biology studies followed by reverse genetics approaches.

3.3 nhaA *and* hfq

The *hfq* overexpression plasmid p42-0347 was introduced into acetate-tolerant *Z. mobilis* strains AcR (Joachimstahl et al. 1998) and ZM4 deletion mutant ZM4DM0117 (Yang et al. 2010a), which overexpress the *nhaA* gene to examine whether or not even higher levels of NaAc tolerance could be achieved (Fig. 3). All the strains grew similarly in RM broth, except that those carrying plasmid DNA had slightly reduced growth rates (Fig. 3a), consistent with previous reports (Yang et al. 2010a,2010b). The combined overexpression of *hfq* and *nhaA,* either in an AcR or in a ZM4DM0117 background, did not augment the NaAc tolerance phenotype (Fig. 3b). ZM4 is unable to grow in RM with 195 mM (16 g/L) NaAc, while strain AcR grows well (Joachimstahl et al. 1998; Yang et al. 2010a) (Fig. 3b). The introduction of *hfq*-overexpressing plasmid p42-0347 can improve the NaAc tolerance of wild-type *Z. mobilis* with 195 mM NaAc (Yang et al. 2010b) but neither the deletion mutant ZM4DM0117 nor acetate-tolerant mutant AcR that both already have enhanced NaAc tolerance through *nhaA* overexpression (Yang et al. 2010a) (Fig. 3b). A similar trend for the growth phenotypes was observed under more inhibitory conditions, i.e., 243 mM (20 g/L) or 364 mM (30 g/L) NaAc for AcR strain containing p42-0347 plasmid. The growth rate of ZM4DM0117 (p42-0347) was approximately one quarter less than that of ZM4DM0117 in RM with 195 mM NaAc (Fig. 3b). In addition, the final culture turbidity of ZM4DM0117(p42-0347) in RM with 195 mM NaAc, as measured by OD_{600nm} units, was also reduced by more than one-fifth to 0.37 ± 0.007 compared to the parental strain ZM4DM0117 (Fig. 3b). This indicates that higher levels of NaAc tolerance are not achieved by combining the two independent *hfq* and *nhaA* overexpression mechanisms for *Z. mobilis* NaAc tolerance.

3.4 himA

Another approach to identifying inhibitor-tolerant gene targets and to better understanding microbial physiology uses targeted mutant library construction and characterization. For example, scientists at NREL and DuPont constructed a transposon mutant library of a xylose-utilizing *Z. mobilis* strain and identified a *himA* gene involved in acetate tolerance of *Z. mobilis* (Viitanen et al. 2009). They further engineered a *himA* markerless mutant with reduced *himA* activity and increased

ethanol production compared to parental strains when cultured in a mixed-sugar medium containing xylose, especially in the presence of acetate (Viitanen et al. 2009).

In a similar approach, scientists at the Energy Biosciences Institute (EBI) constructed a "bar-coded" transposon library of *Z. mobilis*. They have established a pooled transposon library containing insertions in 1,695 different genes from 14,009 transposon insertion mutants that includes most non-essential genes in the *Z. mobilis* genome. In addition, a high-throughput 96-well growth screen has been carried out to determine the inhibitory concentration of various inhibitors and potential fuel molecules. The details about this ongoing project can be accessed at the website: http://www.energybiosciencesinstitute.org/index.php?option=com_content&task=view&id=124&Itemid=20. The gene targets identified through this study will hopefully add more inhibitor-tolerant genes for future metabolic engineering or synthetic biology endeavors.

4 Heterologous Expression for Strain Improvement

Heterologous expression of genomic DNA from resistant microorganisms is another strategy that can be employed for strain development purposes. *Deinococcus radiodurans* is an extremely tolerant microorganism isolated in highly radioactive and extreme environments (White et al. 1999). The *D. radiodurans* IrrE protein was identified as a regulator of *recA* expression (Earl et al. 2002), and its heterogeneous expression in *E. coli* promotes DNA repair and protection against oxidative damage (Gao et al. 2003). Although *D. radiodurans* and *E. coli* are quite different organisms, the *irrE* gene protects *E. coli* against multiple stresses, including oxidative, osmotic, and thermal shocks, and confers greater salt tolerance in plants (Pan et al. 2009). Recently, researchers have shown that the *D. radiodurans irrE* gene also confers improved *Z. mobilis* cell viability, abiotic stress tolerance, and ethanol production (Zhang et al. 2010). Numbers of transcripts for key *Z. mobilis* genes (pyruvate decarboxylase and alcohol dehydrogenase) and their enzyme activities were higher in IrrE-expressing *Z. mobilis* as compared to empty vector control strains (Zhang et al. 2010). These studies and others show the potential for heterogeneous expression to expand the genetic pool for strain improvement.

5 Conclusion and Perspectives

In conclusion, recent studies using *Z. mobilis* as a model indicated that accurate genome annotation is crucial for systems biology studies and, in turn, that the data generated from systems biology studies are important for genome annotation improvements. A paradigm for rapid identification and characterization of process-relevant traits created by classical strain development has been proposed through

the integration of systems biology and next-generation sequencing approaches with genetics tools. This affirms the notion that near-term pathway engineering strategies benefit from a combinatorial approach (Alper and Stephanopoulos 2009) as well as the potential to identify the inhibitor-tolerant gene targets by forward genetics (*hfq* case). Gene targets identified from the approaches above can be extended to other industrial biocatalysts by homolog searching and genetics tools (Yang et al. 2010a, b). The phenotypic trait of the acetate-tolerant AcR mutant is largely due to truncation of the *nhaA* promoter region in the AcR, which suggests that future investigations into transcription unit architecture will be a valuable area to pursue through the application RNA-Seq or tiling array technologies. At the same time, integration of information from other omics platforms such as proteomics and metabolomics will provide a more comprehensive profile for metabolic engineering and modeling (Lee et al. 2010). Finally, regulatory networks need to be taken into consideration to better understand and manipulate microbial physiology. (Alper et al. 2006; Alper and Stephanopoulos 2007; Tyo et al. 2007; Cho et al. 2007).

Acknowledgments The authors would like to thank Meghan M. Drake for her careful review and suggestions. The BioEnergy Science Center is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. This manuscript has been authored by UT-Battelle, LLC, under Contract No. DE-AC05-00OR22725 with the US Department of Energy.

References

Almeida JRM, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349

Alper H, Stephanopoulos G (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab Eng 9:258–267

Alper H, Stephanopoulos G (2009) Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? Nat Rev Micro 7:715–723

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568

Armengaud J (2009) A perfect genome annotation is within reach with the proteomics and genomics alliance. Curr Opin Microbiol 12:292–300

Baudet M, Ortet P, Gaillard JC, Fernandez B, Guerin P, Enjalbal C, Subra G, de Groot A, Barakat M, Dedieu A, Armengaud J (2010) Proteomics-based refinement of *Deinococcus deserti* genome annotation reveals an unwanted use of non-canonical translation initiation codons. Mol Cell Proteomics 9:415–426

Cho BK, Charusanti P, Herrgard MJ, Palsson BO (2007) Microbial regulatory and metabolic networks. Curr Opin Biotechnol 18:360–364

Deanda K, Zhang M, Eddy C, Picataggio S (1996) Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. Appl Environ Microbiol 62:4465–4470

Devos D, Valencia A (2001) Intrinsic errors in genome annotation. Trends Genet 17:429–431

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Applied Microbiol Biotechnol 63:258–266

Earl AM, Mohundro MM, Mian IS, Battista JR (2002) The IrrE protein of *Deinococcus radiodurans* R1 is a novel regulator of *recA* expression. J Bacteriol 184:6216–6224

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM et al (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269:496–512

Gao GJ, Tian B, Liu LL, Sheng DH, Shen BH, Hua YJ (2003) Expression of *Deinococcus radiodurans* PprI enhances the radioresistance of *Escherichia coli*. DNA Repair 2:1419–1427

Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G (2006) Bio-ethanol–the fuel of tomorrow from the residues of today. Trends Biotechnol 24:549–556

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 315:804–807

Jeffries TW (2005) Ethanol fermentation on the move. Nat Biotechnol 23(1):40–41

Jeon YJ, Svenson CJ, Joachimsthal EL, Rogers PL (2002) Kinetic analysis of ethanol production by an acetate-resistant strain of recombinant *Zymomonas mobilis*. Biotechnol Lett 24:819–824

Joachimstahl E, Haggett KD, Jang JH, Rogers PL (1998) A mutant of *Zymomonas mobilis* ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnol Lett 20:137–142

Kadar Z, Maltha SF, Szengyel Z, Reczey K, De Laat W (2007) Ethanol fermentation of various pretreated and hydrolyzed substrates at low initial pH. Appl Biochem Biotechnol 137:847–858

Kerr AL, Jeon YJ, Svenson CJ, Rogers PL, Neilan BA (2010) DNA restriction-modification systems in the ethanologen, *Zymomonas mobilis* ZM4. Appl Microbiol Biotechnol 89:761–769

Kim IS, Barrow KD, Rogers PL (2000) Nuclear magnetic resonance studies of acetic acid inhibition of *rec Zymomonas mobili*s ZM4(pZB5). Appl Biochem Biotechnol 84–6:357–370

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Kouvelis VN, Saunders E, Brettin TS, Bruce D, Detter C, Han C, Typas MA, Pappas KM (2009) Complete genome sequence of the ethanol producer *Zymomonas mobilis* NCIMB 11163. J Bacteriol 191:7140–7141

Lawford HG, Rousseau JD (1993) The effect of acetic acid on fuel ethanol-Production by *Zymomonas*. Appl Biochem Biotechnol 39:687–699

Lawford HG, Rousseau JD (1998) Improving fermentation performance of recombinant *Zymomonas* in acetic acid-containing media. Appl Biochem Biotechnol 70–2:161–172

Lawford HG, Rousseau JD, Tolan JS (2001) Comparative ethanol productivities of different *Zymomonas* recombinants fermenting oat hull hydrolysate. Appl Biochem Biotechnol 91–3:133–146

Lee K, Park J, Kim T, Yun H, Lee S (2010) The genome-scale metabolic network analysis of *Zymomonas mobilis* ZM4 explains physiological features and suggests ethanol and succinic acid production strategies. Microb Cell Fact 9:94

Linger JG, Adney WS, Darzins A (2010) Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*. Appl Environ Microbiol 76:6360–6369

Liu Z, Blaschek H (2010) Biomass conversion inhibitors and in situ detoxification. In: Vertes A, Qureshi N, Yukawa H, Blaschek H (eds) Biomass to biofuels: strategies for global industries. Wiley, West Sussex, p 27

Liu ZL, Moon J (2009) A novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31:345–352

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Applied Microbiol Biotechnol 81:743–753

Liu ZL, Ma M, Song M (2009) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282:233–244

MacLean D, Jones JD, Studholme DJ (2009) Application of 'next-generation' sequencing technologies to microbial genetics. Nat Rev 7:287–296

McMillan JD (1994) Conversion of hemicellulose hydrolyzates to ethanol. In: Himmel ME, Baker JO, Overend RP (eds) Enzymatic conversion of biomass for fuels production, vol 566, ACS Symposium Series., pp 411–437

Metzker ML (2010) Sequencing technologies – the next generation. Nat Rev Genet 11:31–46

Mills T, Sandoval N, Gill R (2009) Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol Biofuels 2:26

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Biores Technol 74:25–33

Pan J, Wang J, Zhou ZF, Yan YL, Zhang W, Lu W, Ping S, Dai QL, Yuan ML, Feng B, Hou XG, Zhang Y, Ma R, Liu T, Feng L, Wang L, Chen M, Lin M (2009) IrrE, a global regulator of extreme radiation resistance in *Deinococcus radiodurans*, enhances salt tolerance in *Escherichia coli* and *Brassica napus*. PLoS One 4:2

Panesar PS, Marwaha SS, Kennedy JF (2006) *Zymomonas mobilis*: an alternative ethanol producer. J Chem Technol Biotechnol 81:623–635

Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287–301

Park JH, Lee SY, Kim TY, Kim HU (2008) Application of systems biology for bioprocess development. Trends Biotechnol 26:404–412

Patnaik R (2008) Engineering complex phenotypes in industrial strains. Biotechnol Prog 24:38–47

Payne SH, Huang ST, Pieper R (2010) A proteogenomic update to Yersinia: enhancing genome annotation. BMC Genomics 11:460

Pienkos PT, Zhang M (2010) Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. Cellulose 16:20

Ranatunga TD, Jervis J, Helm RF, McMillan JD, Hatzis C (1997) Identification of inhibitory components toxic toward *Zymomonas mobilis* CP4(pZB5) xylose fermentation. Appl Biochem Biotechnol 67:185–198

Rogers PL, Jeon YJ, Lee KJ, Lawford HG (2007) *Zymomonas mobilis* for fuel ethanol and higher value products. In: Biofuels, vol 108. Advances in Biochemical Engineering/ Biotechnology. pp 263–288

Seo JS, Chong HY, Park HS, Yoon KO, Jung C, Kim JJ, Hong JH, Kim H, Kim JH, Kil JI, Park CJ, Oh HM, Lee JS, Jin SJ, Um HW, Lee HJ, Oh SJ, Kim JY, Kang HL, Lee SY, Lee KJ, Kang HS (2005) The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. Nat Biotechnol 23:63–68

Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, Hinton JC, Vogel J (2008) Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator. Hfq PLoS Genet 4:8

Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science 315:801–804

Takahashi CM, Takahashi DF, Carvalhal MLC, Alterthum F (1999) Effects of acetate on the growth and fermentation performance of *Escherichia coli* KO11. Appl Biochem Biotechnol 81:193–203

Tsui HC, Leung HC, Winkler ME (1994) Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. Mol Microbiol 13:35–49

Tyo KE, Alper HS, Stephanopoulos GN (2007) Expanding the metabolic engineering toolbox: more options to engineer cells. Trends Biotechnol 25:132–137

Valentin-Hansen P, Eriksen M, Udesen C (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. Mol Microbiol 51:1525–1533

Viitanen PV, Tao L, Knoke K, Zhang Y, Caimi PG, Zhang M, Chou Y, Franden M (2009) Process for the production of ethanol from a medium comprising xylose, employing a recombinant *Zymomonas* strain having a reduced *himA* expression. Patent WO/2009/058938

White O, Eisen JA, Heidelberg JF, Hickey EK, Peterson JD, Dodson RJ, Haft DH, Gwinn ML, Nelson WC, Richardson DL, Moffat KS, Qin H, Jiang L, Pamphile W, Crosby M, Shen M, Vamathevan JJ, Lam P, McDonald L, Utterback T, Zalewski C, Makarova KS, Aravind L, Daly MJ, Minton KW, Fleischmann RD, Ketchum KA, Nelson KE, Salzberg S, Smith HO, Venter JC, Fraser CM (1999) Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science 286:1571–1577

Widiastuti H, Kim JY, Selvarasu S, Karimi IA, Kim H, Seo JS, Lee DY (2010) Genome-scale modeling and in silico analysis of ethanologenic bacteria *Zymomonas mobilis*. Biotechnol Bioeng 108:655–665

Wright JC, Sugden D, Francis-McIntyre S, Riba-Garcia I, Gaskell SJ, Grigoriev IV, Baker SE, Beynon RJ, Hubbard SJ (2009) Exploiting proteomic data for genome annotation and gene model validation in *Aspergillus niger*. BMC Genomics 10:61

Yablonsky MD, Goodman AE, Stevnsborg N, Delima OG, Demorais JOF, Lawford HG, Rogers PL, Eveleigh DE (1988) *Zymomonas mobilis* CP4: a clarification of strains *via* plasmid profiles. J Biotechnol 9:71–79

Yang S, Pappas KM, Hauser LJ, Land ML, Chen G-L, Hurst GB, Pan C, Kouvelis V, Typas M, Pelletier DA, Klingeman DM, Chang Y-J, Samatova NF, Brown SD (2009a) Improved genome annotation for Zymomonas mobilis. Nat Biotechnol 27:893–894

Yang S, Tschaplinski TJ, Engle NL, Carroll SL, Martin SL, Davison BH, Palumbo AV, Rodriguez M Jr, Brown SD (2009b) Transcriptomic and metabolomic profiling of *Zymomonas mobilis* during aerobic and anaerobic fermentations. BMC Genomics 10:34

Yang S, Land ML, Klingeman DM, Pelletier DA, Lu T-YS, Martin SL, Guo HB, Smith JC, Brown SD (2010a) Paradigm for industrial strain improvement identifies sodium acetate tolerance loci in *Zymomonas mobilis* and *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 107:10395–10400

Yang S, Pelletier DA, Lu TY, Brown SD (2010b) The *Zymomonas mobilis* regulator *hfq* contributes to tolerance against multiple lignocellulosic pretreatment inhibitors. BMC Microbiol 10:135

Zhang M, Eddy C, Deanda K, Finkestein M, Picataggio S (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science 267:240–243

Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S (2003) Global analysis of small RNA and mRNA targets of Hfq. Mol Microbiol 50:1111–1124

Zhang Y, Ma RQ, Zhao ZL, Zhou ZF, Lu W, Zhang W, Chen M (2010) *irrE*, an exogenous gene from *Deinococcus radiodurans*, improves the growth of and ethanol production by a *Zymomonas mobilis* strain under ethanol and acid stresses. J Microbiol Biotechnol 20:1156–1162

Mechanisms and Applications of Microbial Solvent Tolerance

Mark Taylor, Jean-Baptiste Ramond, Marla Tuffin, Stephanie Burton, Kirsten Eley, and Don Cowan

Contents

1 Introduction 178
2 Biological Application and Relevance 182
3 Biochemical and Physiological Mechanisms of Solvent Tolerance 184
3.1 Membrane Adaptation 185
3.2 Solvent Exclusion 186
3.3 Regulation of Response 189
4 Improving the Solvent-Tolerant Phenotype in Microbial Hosts 190
4.1 Yeasts 190
4.2 Mesophilic Bacteria 191
4.3 Thermophilic Bacteria 193
4.4 Microalgae and Cyanobacteria 194
4.5 Microbial Communities and Consortia 195
5 Applications for Biofuels 196
6 Conclusion and Perspectives 198
References 199

M. Taylor
Department of Biotechnology, Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Bellville 7535, Cape Town, South Africa

TMO Renewables, The Surrey Research Park, Guildford, Surrey GU2 7YF, UK

J.-B. Ramond • M. Tuffin • D. Cowan (✉)
Department of Biotechnology, Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Bellville 7535, Cape Town, South Africa
e-mail: dcowan@uwc.ac.za

S. Burton
Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology, Bellville Campus, Bellville, 7535 Cape Town, South Africa

K. Eley
TMO Renewables, The Surrey Research Park, Guildford, Surrey GU2 7YF, UK

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_8,

Abstract Biofuels currently feature heavily on scientific, social, and political agenda, and particular focus is reserved for liquid fuels that may act as a substitute or blending agent for petroleum. Many pertinent questions arise when a thorough analysis of the feasibility of liquid alcohol fuels is performed. The focus of this chapter is to analyze our current understanding of the mechanisms that contribute to one of these issues, namely, how can an organism adapt to tolerate usually cytotoxic levels of solvent or alcohol. A considerable volume of research has contributed to our current understanding of the general cellular mechanisms and physiological responses that occur in response to solvent shock. This foundation of knowledge has subsequently allowed a deeper understanding as to adaptive changes responsible for solvent-tolerant phenotypes in mutant progeny. Here we review a number of more common cell responses to solvents, with particular focus on alcohol tolerance, with the aim to place this topic in its correct context as a central theme in understanding the microbial physiology of biofuel production.

1 Introduction

Organic solvents are important chemicals since they (i) are commonly used in laboratories and in many chemical and pharmaceutical processes, (ii) constitute by-products or wastes of various industrial processes, and (iii) are a principal focus as alternative fuels. Many solvents used in industrial manufacturing are environmental contaminants (e.g., polyaromatic hydrocarbons [PAH]) and may be hazardous components of common industrial wastes (e.g., phenolic compounds). Solvents of this kind require removal or deconstruction into less harmful compounds, a process generally referred to as bioremediation. Moreover, the ongoing interest in alternatives to classic oil-based fuels and the global concern over their greenhouse gas (GHG) emissions have led to the development of the bioproduction of fuel(s) and chemicals from "environmental friendly" renewable sources (Lynd et al. 2008; Taylor et al. 2009).

Organic solvents, when produced in sufficient quantities biologically or when present as permanent or transient contamination in the external environment, pose a significant biological threat. Exposure to organic solvents has multiple debilitating effects on the cell, principally involving interference with cell membrane integrity and function. With the exception of a few well-characterized processes, such as the fermentative production of ethanol, such effects have always been a major disadvantage in the development of biotechnological processes centered on organic solvent production. In spite of this limitation, the development of microbial technologies to produce and/or degrade solvents remains a major research focus, as summarized for the readers' convenience in Table 1 (Lynd et al. 2008; Prpich and Daugulis 2005; Sardessai and Bhosle 2002; Taylor et al. 2009; Zverlov et al. 2006). A correlation between solvent hydrophobicity and antimicrobial properties has been established (Isken and de Bont 1998) and is used as an assay of solvent tolerance. Microorganisms that are able to survive and thrive in the presence of high

Table 1 Solvent production by strains of commercial or research value

Microorganisms	Strain	Solvent production	Yield (g biofuel/g carbon source) or titer ([m]g biofuel/L culture)	References
Pseudomonas putida	TPL3	Phenol	0.031 gg^{-1}	(Waege et al. 2010; Wierckx et al. 2008; Wierckx et al. 2005)
Escherichia coli	SZ420	Ethanol	0.32–0.37 gg^{-1}	(Atsumi et al. 2008; Cann and Liao 2010; Clomburg and Gonzalez 2010; Doan et al. 2009; Ingram et al. 1998; Inui et al. 2008; Kim et al. 2007; Lee et al. 2008a; Shen and Liao 2008; Trinh et al. 2008; Wang et al. 2008; Zhang et al. 2008; Zhou et al. 2008)
	SE2378	1-Butanol	0.41 gg^{-1}	
	KO11	Propanol	0.48 gg^{-1}	
	JCL187	Pentanol	0.03 gg^{-1}	
	BUT2	Isopropanol	0.02 gg^{-1}	
	MG1655	Isopentanol	0.11 gg^{-1}	
	Recombinant DH10B	2-Methyl-1-butanol	0.14 gg^{-1}	
	Recombinant BW25113	3-Methyl-1-butanol, fatty alcohols	0.14 gg^{-1}	
	JCL16		0.006 gg^{-1}	
	Recombinant XL1 blue		0.17 gg^{-1}	
			0.11–13 gg^{-1}	
			Trace	
Saccharomyces cerevisiae	Various industrial strains	Ethanol	~0.46 gg^{-1}	(Lee et al. 2008a)
Klebsiella oxytoca	Recombinant M5A1	Ethanol	0.5 gg^{-1}	(Burchhardt and Ingram 1992)
Zymomonas mobilis	Recombinant 8938	Ethanol	0.49 gg^{-1}	(Rao and Jones 2004; Yanase et al. 2005)
Zymomonas sp.	B806	1-Butanol	13.5 mgL^{-1}	
		2-Methyl-1-butanol	394 mgL^{-1}	
		3-Methyl-1-butanol	113 mgL^{-1}	
		2-Methyl-2-butanol, propanol	40 mgL^{-1}	
		Phenol	15 mgL^{-1}	
			112 mgL^{-1}	
Clostridium acetobutylicum	Recombinant ATCC 824	Total solvent (ABE)[a]	~20 gL^{-1}	(Ezeji et al. 2010; Ezeji et al. 2007b; Keis et al. 2001b; Keis et al. 2001a; Zverlov et al. 2006)
		Butanol	~13 gL^{-1}	
C. saccharobutylicum	ATCC BAA-117^T	Butanol	11–11.5 gL^{-1}	
C. beijerinckii	Recombinant NCIMB 8052	Total solvent (ABE)	~29 gL^{-1}	
		Butanol	~19 gL^{-1}	
C. ljungdahlii	ATCC 55383	Ethanol	600 mgL^{-1}	

(continued)

Table 1 (continued)

Microorganisms	Strain	Solvent production	Yield (g biofuel/g carbon source) or titer ([m]g biofuel/L culture)	References
C. autoethanogenum	DSMZ 10061	Ethanol	240 mgL^{-1}	
C. thermocellum	ATCC 27405	Ethanol	1.2 gL^{-1}	(Lynd et al. 1989; Wiegel et al. 1979)
C. thermohydrosulfuricum	JW102	Ethanol	1.3–2.5 gL^{-1}	
	E100-69	Ethanol	1.3 gL^{-1}	
Botryococcus braunii	Var. Showa	Various hydrocarbons	~0.95 gL^{-1}	(Metzger and Largeau 2005)
Co-culture:	WD 161	Total solvent (ABE)	9.7 gL^{-1}	(Tran et al. 2010)
Bacillus subtilis	TISTR 1032			
Clostridium butylicum				
Aeromonas sp.	ATCC 29063	Phenol	~0.38 gL^{-1}	(Carman and Levin 1977; Chen and Levin 1975)
Tolumonas auensis	TA4	Toluene	6–338 mgg^{-1}	(Fischer-Romero et al. 1996)
Thermoanaerobacter ethanolicus	JW200	Ethanol	0.29–0.42 gg^{-1}	(Hild et al. 2003; Lacis and Lawford 1992; Peng et al. 2008a; Taylor et al. 2009)
T. saccharolyticum	JW/SL-YS485	Ethanol	0.38 gg^{-1}	
T. mathranii	BG1L1	Ethanol	0.39–0.42 gg^{-1}	
Geobacillus stearothermophilus	LLD15	Ethanol	0.3 gg^{-1}	(San Martin et al. 1992; Taylor et al. 2009)
G. thermoglucosidasius	Recombinant NCIMB 11955	Ethanol	0.44 gg^{-1}	

[a]*ABE* Acetone-butanol-ethanol

solvent concentrations are considered as "extremophiles" and can either be isolated from natural environments or engineered to be so (Clomburg and Gonzalez 2010; Essam et al. 2010; Fischer-Romero et al. 1996; Isken and de Bont 1998; Lee et al. 2008a; Wierckx et al. 2008).

The ability to isolate or develop extremely solvent-tolerant bacteria and to study their physiology in order to understand how they are able to survive conditions that are lethal to "normal" prokaryotes has thrown light on many synergistic mechanisms that maintain intracellular homeostasis in these organisms. However, the development of solvent-resistant organisms and their integration into biotechnology processes have remained largely undeveloped in spite of a growing understanding of the unique physiology and solvent tolerance of such strains. Whole-cell biotransformations are often favored over enzymatic systems as they allow multiple and complex enzymatic reactions and avoid the use of expensive cofactors or coenzymes (e.g., ATP, NAD(P)H); (Heipieper et al. 2007). Nevertheless, the issues of solvent tolerance have recently been thrown back into the spotlight as a result of the rapid resurgence of interest in solvent-related processes, such as the bioproduction of alcohols.

The active pursuit of suitable renewable alternatives to oil-based fuels has become a global socioeconomic priority, the dominant interest being in developing biotechnological ethanol production destined for an alcohol–petroleum blend that can be used in current or modified combustion engines. Conventional ethanol production for such purpose is well established and supports the current global demand for transportation ethanol. To date, the biological production of ethanol for fuel supplementation has focused on conventional fermentation with *Saccharomyces cerevisiae* (Lin and Tanaka 2006; van Zyl et al. 2007), a process that delivers economical ethanol yields while being highly ethanol-tolerant. However, with concerns over the impacts that a renewable fuel economy may have on food security and agricultural land use, developers have sought to circumvent any potential conflict involving the use of consumable (food-grade) carbohydrate feedstocks. The current focus is on the development of processes based on lignocellulosic fermentation and requires a degree of catabolic versatility beyond that possessed by wild-type strains of *S. cerevisiae*. Concurrently, there has been a realization that a much wider range of potentially valuable metabolites can be produced from this carbohydrate source, such as butanol, branched-chain alcohols, acetone, etc. (Ezeji et al. 2007a; Fischer et al. 2008; Rogers et al. 2007).

Unfortunately, a diverse catabolic nature and the ability to produce a single end point metabolite do not appear to come hand in hand. In consequence, researchers have sought to engineer fermentative versatility in ethanologenic organisms, such as *Z. mobilis* (Buchholz and Eveleigh 1990; Lin et al. 2005; Mohagheghi et al. 2002) and ethanol production capability in more catabolically diverse organisms, such as *E. coli* (Alterthum and Ingram 1989; Bothast et al. 1999; Ingram et al. 1987; Wang et al. 2008). In both cases, the development of an organism capable of producing high solvent yields induces a parallel requirement for tolerance to previously cytotoxic concentrations of solvent.

2 Biological Application and Relevance

Ethanol (C_2H_5OH) is a common fuel oxygenate in reformulated gasoline. The bioproduction of ethanol is now divided into first- and second-generation processes (Taylor et al. 2009). First-generation ethanol is derived from food crops, such as starch or sugar cane (Lee et al. 2008a; Taylor et al. 2009), *via* the anaerobic fermentation of the constituent sugars, sucrose and glucose. Many microorganisms are able to ferment sugars and produce bioethanol, the most widely known being the yeast *Saccharomyces cerevisiae*. This technology is mature and commercially developed. However, even though first-generation bioethanol is renewable (i.e., the feedstocks can be regrown), the requirement for expensive feedstock plantations can negatively impact on product costs, and technology has been criticized for affecting food prices. In light of the "fuel *versus* food" debate and its negative connotations in developing countries, there are substantial pressures to find alternatives to the first-generation process (Ni et al. 2007; Taylor et al. 2009). Plant biomass, as a potentially abundant resource of carbon, in the form of lignocellulose, can be used in biofuel production. This concept has formed the basis of a great deal of recent research and numerous review articles (Dellomonaco et al. 2010; Lee et al. 2008a; Ni et al. 2007; Taylor et al. 2009). Lignocellulosic biomass accounts for about 50% of biomass in the world. The principal challenge in its use for bioethanol production processes is the achievement of high yields and hence a reduction of ethanol prices to a competitive level with petroleum. Such production is considered as a second-generation biofuel technology (Taylor et al. 2009). However, the production of bioethanol from lignocellulosic materials is substantially more complicated than from simple C6 monosaccharides; lignocellulose is a complex molecule composed of cellulose, hemicellulose, and lignin, which require a degree of depolymerization and deconstruction before fermentation is possible. Cellulose and hemicellulose are polymers of both C6 and C5 sugar molecules, and their complete hydrolysis leads to the production of monomeric sugars; only the C6 component of which can be used for ethanol production by conventional fermentation (Sun and Cheng 2002). The development of strains able to produce bioethanol from non-C6 sugars is thus of great interest. This has included the metabolic improvement of well-known, existing, ethanologenic mesophilic strains (e.g,. *S. cerevisiae*, *Zymomonas mobilis*, *E. coli,* and *Klebsiella oxytoca*) and thermophilic strains (e.g., *Thermoanaerobacter mathranii*, *Thermoanaerobacterium saccharolyticum*, *Geobacillus thermoglucosidasius*) (Burchhardt and Ingram 1992; Clomburg and Gonzalez 2010; Dien et al. 2003; Lee et al. 2008a; Taylor et al. 2009; Yanase et al. 2005).

Butanol ($C_4H_{10}O$) is generally regarded as a more valuable biofuel than ethanol. This is due to its physical properties, particularly its higher energy content and higher boiling point than ethanol. Moreover, as the vapor pressure of 1-butanol is 11 times lower than that of ethanol under normal atmospheric conditions, it offers significant safety advantages as a transportation fuel. Butanol is also used in many other industries, such as the food and plastic sectors (Zverlov et al. 2006). The

bioproduction of 1-butanol consists of biphasic fermentation, where acetic and butyric acids are produced during an acidogenic phase, followed by their conversion during a solventogenic phase into acetone and butanol. Thus, anaerobic bacterial fermentations typically lead to the production of n-butanol, with acetone as a second major product and ethanol as a minor product. This process is called the AB (acetone–butanol) or ABE (acetone–butanol–ethanol) fermentation and is well characterized in the anaerobic bacterial genus *Clostridium* (Ezeji et al. 2007a, 2010; Tran et al. 2010; Zverlov et al. 2006).

Solventogenic *Clostridium* spp. ferment glucose, sucrose, and starch via the Embden–Meyerhof pathway as well as glycerol and other hexoses, pentoses, and oligosaccharides. A potential advantage of using AB(E) microorganisms rather than established ethanologens such as *Saccharomyces* or *Zymomonas* is that they are able to produce a wider range of solvent products from a wide variety of substrates, including hemicellulose-derived pentose sugars. This concept led to the construction of eight industrial AB fermentation plants in the former USSR; some of these were still in use in the 1980s (Zverlov et al. 2006). The industrially viable bioproduction of butanol is however currently limited by the cost of substrates, by the toxicity of butanol to fermenting microorganisms (which leads to low butanol yields), and by the use of dilute sugar feedstocks, which necessitates large fermentation volumes (Ezeji et al. 2007b; Zverlov et al. 2006). Nevertheless, industrial plants in the former USSR have proved that the use of (i) continual fermentation, (ii) stringent sterilization (to decrease the incidence of bacteriophages), (iii) the replacement of starch by agricultural waste materials, (iv) the use of pentose hydrolyzates and hexoses, and (v) integration of the ABE-producing facility in a biorefinery concept (i.e., possible use of by-products) could lead to an economically viable AB(E) bioproduction (Zverlov et al. 2006).

Various *Clostridium* species, particularly *C. acetobutylicum*, are capable of 1-butanol synthesis. However, the generally poor solvent resistance of these and most other bacteria is a major limiting factor in the profitability of biobutanol production (Ezeji et al. 2010). Genetic manipulation of these microorganisms may lead to the development of hyperbutanol-producing strains (Ezeji et al. 2007a). A number of different approaches recently reviewed by Ezeji and colleagues (Ezeji et al. 2007a) have been used to improve the biobutanol production of solventogenic *Clostridium* strains. These include (1) mutagenesis and selection of more solvent-tolerant variants, (2) fermentation and process developments that reduce solvent exposure or in some way offer "protection" to the actively growing cultures, and (3) site-directed recombinant technologies. Separate approaches to circumvent the poor tolerance to butanol of *Clostridium* spp. have involved coculture with other mesophilic strains and gene expression in recombinant hosts. A coculture of *Bacillus subtilis* and *Clostridium butylicum* was reported to enhance the production of ABE from soluble and cassava starch by up to 6.5-fold when compared to pure cultures of *Clostridium* (Tran et al. 2010). The isolation and expression of the gene set required for butanol production in *E. coli* have also been reported. This approach has the additional advantage that *E. coli* can tolerate 1-butanol up to a concentration of 1.5%, is amiable to genetic alteration, and does not generate by-products, such as

butyrate, acetone, and ethanol, thus potentially butanol yields through the reduction of "side-product" formation (Atsumi et al. 2008). The other solvent produced from the AB(E) fermentation, acetone ($(CH_3)_2CO$), is widely used for cleaning purposes, as an industrial solvent, and as a precursor in polymer synthesis. While acetone is not the major product in ABE fermentations, it represents around 30% of the total solvent production in industrial chemical plants (Zverlov et al. 2006) and may be a future target for production.

Phenolic compounds such as phenol and chlorophenols are widely used in chemical and pharmaceutical industries; many are toxic and have been listed as priority pollutants by the USA Environmental Protection Agency (EPA). Phenol (C_6H_5OH) is an aromatic organic compound naturally found in the environment but for which levels have increased due to anthropogenic activities, essentially related to the petroleum industry and to plastic and/or resin production. Due to their water solubility, phenol and phenolic compounds are general aquatic ecosystem contaminants. Phenol has been found to be produced naturally by solvent-tolerant microorganisms (Chen and Levin 1975; Fischer-Romero et al. 1996; Rao and Jones 2004) and by engineered microorganisms (Wierckx et al. 2005, 2008), which are of particular interest to biosynthesize and degrade toxic compounds to other less harmful products. A number of *Pseudomonas putida* strains have been found to tolerate phenol, and an engineered *P. putida* S12 strain, with an introduced tyrosine phenol-lyase (TPL)-encoding gene (*tpl*), was able to convert glucose into phenol with a yield of 7% mol/mol (Wierckx et al. 2005). Transcriptomic analysis has shown that the enhanced phenol production of *P. putida* S12TPL3 was linked with the tyrosine biosynthetic pathway (i.e., to an upregulation of tyrosine biosynthetic genes) to such an extent that TPL activity was the bottleneck for phenol bioproduction (Wierckx et al. 2008). These studies suggest that *P. putida* S12TPL3 could be used in industries as a biocatalyst for the conversion of glucose into phenols.

The solvents discussed above represent the major current focus of research and development. However, our knowledge of the great metabolic diversity in microorganisms suggests that organisms may be found which are capable of producing or catabolizing other solvent-like compounds. We note that a new organism, *Tolumonas auensis* gen. nov., sp. nov., was isolated from a toluene-contaminated environment and showed the ability to transform phenylalanine, phenyllactate, phenylpyruvate, and phenylacetate into toluene under both aerobic and anaerobic conditions (Fischer-Romero et al. 1996). The future possibility of toluene bioproduction therefore exists.

3 Biochemical and Physiological Mechanisms of Solvent Tolerance

The primary site of interaction between the organism and a solvent is the cell membrane (Ingram 1990; Isken and de Bont 1998; Ramos et al. 1997, 2002; Sardessai and Bhosle 2002; Sikkema et al. 1995). Solvents disrupt membrane

fluidity and structure by partitioning into the lipid bilayer (Ramos et al. 2002; Sardessai and Bhosle 2002; Sikkema et al. 1994). The toxicity of a solvent correlates to its log*P* value, which is defined as the partition coefficient of a particular solvent in an equimolar solution of octanol and water (Sikkema et al. 1995). Generally, the lower the log*P* value, the greater the polarity of the solvent molecule, and hence the greater its ability to mediate a toxic response through membrane partitioning (Isken and de Bont 1998; Ramos et al. 2002; Sikkema et al. 1994; Zahir et al. 2006). A log*P* value below four is generally considered toxic to cells since the solvent will readily partition into the cell when suspended in an aqueous phase, thus exposing the cells to high concentrations of solvent and impairing membrane function (Ramos et al. 2002). The general impression is that many of the solvents considered as valuable biofuels or common environmental pollutants are quite toxic. For example, ethanol has a log*P* value of –0.31, and butanol a value of 0.8, whereas toluene and benzene have values of 2.5 and 2, respectively. Beyond the ability of the cell envelope to act as a physical barrier, a number of other general responses that assist an organism in resisting the toxic effects of solvents have been described. Here, we present physiological mechanisms associated with tolerance to organic solvents.

3.1 Membrane Adaptation

The first line of cellular defense is the cell envelope, which, in this instance, acts as a physical barrier to solvent penetration (Ramos et al. 1997). However, because of the physiochemical nature of solvents, the phospholipid bilayer is vulnerable to disruption and solubilization via nonspecific permeabilization. These physical changes effectively interfere with membrane barrier function as well as render the membrane defunct as a matrix for enzymes, proteins, and energy transduction (Ding et al. 2009; Ezeji et al. 2010; Zgurskaya et al. 2009). In order to minimize these effects, biological membranes are capable of adaptation and modification.

The majority of studies on membrane adaptation have focused on a limited number of solvent-tolerant Gram-negative bacteria, which include several well-characterized *Pseudomonas* spp. (Pinkart and White 1997; Ramos et al. 2002; Sardessai and Bhosle 2002; Segura et al. 2004). The modifications reported in these strains cumulatively increase membrane rigidity and decrease permeability, including *cis–trans* isomerization (Wang et al. 2009), decreased cell surface hydrophobicity (Ramos et al. 2002), and changes in the chemical composition or proportions of membrane lipids and proteins (Segura et al. 2004).

The *cis–trans* isomerization of unsaturated fatty acids is utilized as a rapid yet effective response to transient solvent exposure and is particularly effective in resisting solvent concentrations that exceed the permissive concentration for growth and *de novo* fatty acid synthesis (Heipieper et al. 2003). The double bonds within unsaturated fatty acids undergo isomerization, altering from the *cis* to *trans* conformation. The key difference between these two physiochemical states

lies in the spaciophysical orientation of the different isomeric forms. The *cis* isomer results typically in a kinked fatty acid conformation that imparts high degrees of fluidity to the membrane, whereas the *trans* orientation is linear, in much the same conformation as a saturated fatty acid. The enhanced packing of the fatty-acyl chains decreases membrane fluidity and improves the solvent resistance phenotype (Pinkart and White 1997; Ramos et al. 2002). Other changes in the chemical composition of membrane fatty acids include increases in the localized concentration of saturated fatty acids, which have very similar effects on membrane fluidity as *cis–trans* isomerization (Segura et al. 2004). This response is only effective in actively growing cells as it is dependent on active cell division and fatty acid synthesis and so is considered a long-term response to solvent/stress exposure (Segura et al. 2004). At concentrations of solvent beyond levels that permit active growth, this mechanism has been shown to be downregulated in a number of strains (Ramos et al. 1997).

In yeast cells, damage by ethanol has been shown to principally occur at the level of the membrane and manifest in growth inhibition, reduced fermentative ability, reduced viability, reduced respiration, lipid modification, reduced proton motive force, increased membrane permeability, and reduced intracellular pH (Attfield et al. 1997). However, ethanol-sensitive phenotypes have also been reported to occur in response to deletions in the *aro* gene clusters (involved in amino acid biosynthesis) (Yoshikawa et al. 2009), the *vma* and *vps* genes (involved in vacuolar function and transportation; Fujita et al. 2006; Yoshikawa et al. 2009) and *btn*2 (involved in v-SNARE binding and protein transference from the late endosome to the Golgi body), indicating that solvent response mechanisms in yeast go beyond membrane adaptation, chaperone upregulation, and other aspects of the general cell "stress response" (Zhao and Bai 2009). In general, however, it is understood that cell membrane integrity is key to an organism's ability to tolerate high levels of exogenous ethanol, where the integrity is maintained under these conditions by an increase in the degree of polyunsaturated fatty acids (PUFA, principally palmitoleic and oleic mono-UFAs, the synthesis of which is regulated by the *ole*1 gene product, a desaturase; Mishra and Kaur 1991). More recent evidence has suggested that oleic acid principally mediates ethanol tolerance rather than a cumulative effect brought on by a higher desaturation index (You et al. 2003). A general decrease in the levels of sterols in the cell membrane and increases in the levels of ergosterol and lanosterol and increase in membrane ATPase activity have also been reported as responses to high concentrations of ethanol (Aguilera et al. 2006; Alexandre et al. 1994; Koukkou et al. 1993).

3.2 Solvent Exclusion

The ability to actively exclude solvents via an efflux solvent system has long been understood to play a major role in cell homeostasis, specifically with regard to exposure to solvents and other toxic agents, such as antibiotics and antimicrobials

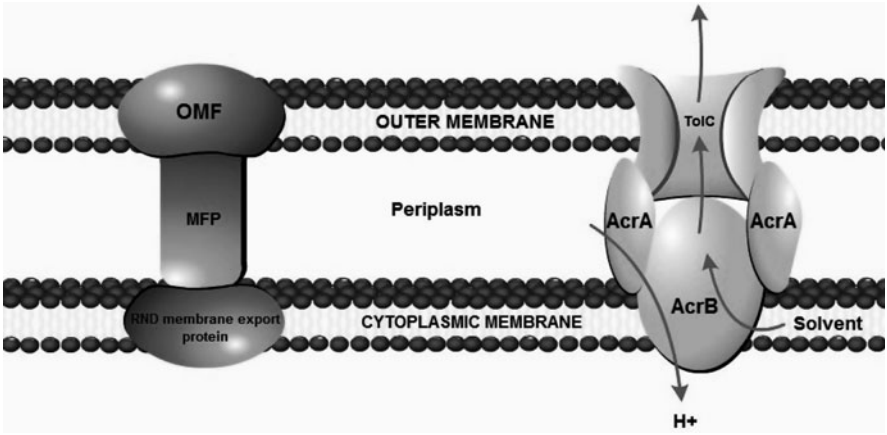

Fig. 1 A general schematic of the RND-type efflux pumps of Gram-negative bacteria and the AcrAB–TolC efflux pump of *E. coli*. RND pumps actively export solvents across the cytoplasmic and outer membranes. They consist of three general components: a cytoplasmic membrane-bound export protein that is an effective energy-dependent pump, a membrane fusion protein (MFP), and an outer membrane-associated protein (OMP). In *E. coli*, the most important solvent efflux pump is the AcrAB–TolC system wherein TolC performs the role of the OMP; AcrA, the MFP; and AcrB is a cytoplasmic bound proton pump. See in text description for more details

(Isken and de Bont 1998; Li and Poole 1999; Li et al. 1998; Ramos et al. 1998). Although multiple efflux mechanisms have been described from a variety of bacteria, all solvent-associated systems described thus far belong to the resistance nodulation cell division (RND) family and predominantly occur in Gram-negative bacteria (Ramos et al. 2002; Sardessai and Bhosle 2002). The general structures of RND-type efflux mechanisms and a brief description of component function, with the AcrAB–TolC efflux pump found in *E. coli* given as an annotated example, are shown in Fig. 1.

E. coli is arguably the best physiologically understood Gram-negative bacterium but is not generally regarded as particularly solvent-tolerant (Ramos et al. 2002; Sardessai and Bhosle 2002; White et al. 1997). In fact, the majority of strains will only tolerate solvents that possess log*P* values > 4. However, certain mutant strains of the *E. coli* strain JA300, which have been described as naturally ampicillin- and chloramphenicol-resistant, have also been shown to have inherited solvent tolerance along with the ability to survive in the presence of these antibiotics (Aono et al. 1995; Aono et al. 1994). It is now understood that the empirical observations of resistance inherited in these antibiotic resistant strains are not coincidental but result from the close links between the mechanisms for both phenotypes in *E. coli*. These are now understood to be the result of the well-characterized tripartite AcrAB–TolC exclusion pump (Aono et al. 1998; Fralick 1996; Zgurskaya and Nikaido 1999a, b; Zgurskaya et al. 2009).

Within the AcrAB–TolC expulsion pump, AcrB serves as the cytoplasmic membrane-bound protein that mediates the active exclusion of toxic molecules.

The crystal structures of these proteins have revealed that three monomeric proteins of AcrB form an integral protein complex that spans the cytoplasmic membrane (Zgurskaya et al. 2009). Each monomer has 12 membrane-spanning helices and a large periplasmic domain which folds to reveal the pore or porter domain, which plays a role in substrate specificity. The monomers of AcrB can exist in three conformational states, and the cyclic transition from state to state is responsible for the pumping mechanism of AcrB, which is driven by the proton motive force. The active site of AcrB contains multiple phenylalanine residues which bind a wide diversity of substrates and explain the multifunctionality of this efflux system (Touze et al. 2004; Zgurskaya and Nikaido 1999a).

The membrane fusion protein or AcrA (recently also referred to as a periplasmic adaptor protein) has four distinct domains: a membrane-proximal domain, a β-barrel, a lipoyl domain, and an α-helical coiled coil hairpin. All of these elements are linked through a series of hinges and β-ribbons which collectively allow a degree of flexibility and up to four different conformational states (Touze et al. 2004; Zgurskaya and Nikaido 1999a; Zgurskaya et al. 2009). The function of AcrA is principally to coordinate the operation of the inner and outer membrane portions of the RND efflux pump. The outer membrane protein TolC is trimeric with each protomer consisting of a β-barrel domain that anchors to the outer membrane and 12 coiled coils which project into the cytoplasm. Both domains form a narrow tunnel-like structure which is thought to be able to contract in a peristaltic motion in order to assist substrate passage through the molecularly distant region of the periplasm and through the outer membrane (Ramos et al. 2002; Touze et al. 2004).

The other prokaryote species which has been widely studied as a model for toxic compound resistance is *Pseudomonas putida* (Inoue et al. 1991; Ramos et al. 1995, 1997, 2002; Sardessai and Bhosle 2002), strains of which have been reported to be resistant to solvents, such as xylene and toluene (Choi et al. 2008; Mosqueda et al. 1999; Ramos et al. 1998). Studies on the mutant progeny of *P. putida*, most significantly the DOT-T1E and GM73 strains, have revealed the most information with regard to solvent tolerance and efflux systems in this species (Kim et al. 1998; Rodriguez-Herva et al. 2007; Rojas et al. 2001). Such studies have revealed the importance of the *srp*ABC gene cluster (solvent-resistant pump) (Sun and Dennis 2009) and the *ttg*ABC genes associated with toluene, styrene, xylene, ethylbenzene, and propylbenzene tolerance (Mosqueda and Ramos 2000; Rojas et al. 2001); both of them show a degree of similarity (amino acid sequence level) to the *acr*ABC operon (Ramos et al. 2002). Unsurprisingly, the structural organization of TtgABC and SrpABC closely mimics that of the AcrABC proteins as assembled in the periplasm (Fig. 1). Other solvent efflux systems in *P. putida* have been described, such as TtgDEF in strain DOT-T1E, which is involved in toluene degradation and only transports styrene and toluene (Mosqueda and Ramos 2000; Rojas et al. 2001), and TtgGHI, a third solvent efflux system with an identical range of function compared to TtgABC (Rojas et al. 2001). The *ttg* and *srp* efflux pumps have a degree of similarity to the MexAB–OprM antibiotic exclusion system described in *P. aeruginosa*, and a wide degree of cross-functionality has been described for all of these exclusion systems (Li and Poole 1999; Li et al. 1998).

3.3 Regulation of Response

High degrees of regulation have been reported for the various systems of solvent tolerance, reflecting the need for a quick and effective response to the changing external environment (Junker and Ramos 1999; Ramos et al. 2002). For example, the activity of *cis–trans* isomerase, which is a cofactor-independent enzyme of 87 kDa, responds to a number of detrimental external stimuli, such as heat shock, antibiotic exposure, heavy metal exposure, and exposure to high concentrations of salts (Heipieper et al. 2003). This gene is moderately rare and is found only on a few microbial genomes from the genera *Pseudomonas*, *Vibrio*, and *Shewanella* (Heipieper et al. 2003). With such a broad range of stimuli, this is considered a general stress response rather than a solvent-specific action. In *P. putida*, although constitutively expressed, exposure to solvent has been reported to elicit a membrane response that results in an increased degree of fatty acid isomerization in the membrane within 5 min (de Carvalho et al. 2004; Ramos et al. 1997). This quick response to solvent in the external environment in this strain indicates that the transcriptional and translational regulation of *cis–trans* isomerase is tightly controlled.

It has been shown that constitutive expression of *P. putida cis–trans* isomerase is a result of a constitutively expressed *rpo*D promoter sequence upstream of the gene (Fujita et al. 1995; Yamamoto et al. 2000). The enzyme is found in the cytosol and periplasm, although it requires a membrane-bound phospholipase and a cytochrome c-like protein in order to function on membrane-integrated lipids (Heipieper et al. 2003). There is some speculation as to the specific mechanism of Cti regulation (Heipieper 2005; Heipieper et al. 2003), and several models that suggest that regulation is based on the physical accessibility of the enzyme to the fatty acids integrated within the membrane have been proposed. One prominent hypothesis states that "activation" of the enzyme only occurs when the membrane is fluid, which, in turn, leads to a greater degree of accessibility to the double bonds of membrane-integrated fatty acids. The transformation of *cis* to *trans* state can then occur, reducing fluidity and hence excluding the enzyme from the membrane (Heipieper 2005; Heipieper et al. 2003).

For regulation of expression of efflux pump proteins, the primary regulator of the *acr*AB operon is the AcrR protein derived from the *acr*R gene, which is what lies upstream of *acr*AB (Ramos et al. 2002). The protein AcrR is self-regulating and is a member of the TetR family of regulators, but *acr*AB can also be transcriptionally regulated by MarR, a regulator of the multiple antibiotic resistance operon MarAB. Regulation may also occur under general stress conditions in the absence of AcrR (Ramos et al. 2002). The *ttg* gene clusters have each been found to be a single operon that is controlled by an adjacent gene and its product (TtgR, TtgT, and TtgV, respectively, for *ttg* ABC, DEF, and GHI) much in the same way as described for *acr*AB (Ramos et al. 2002).

4 Improving the Solvent-Tolerant Phenotype in Microbial Hosts

Although solvent-tolerant organisms can be isolated from natural environments using classical isolation/cultivation approaches coupled with suitable selective pressures, the development of a feasible bioprocess often requires multiple desirable phenotypes in a single strain. For example, it is increasingly apparent that in order to develop an economically viable second-generation bioethanol process, a homoethanologenic strain with a broad catabolic spectrum and high solvent tolerance is required. Such synergy does not occur readily, and so in practice, organisms with moderate capacities for each of the particular phenotypes are sought, and genetic engineering is subsequently employed to selectively enhance each quality to the desired level. In the following section, we aim to review the advances made in the development of solvent tolerance in a number of industrially pertinent strains. Such advances have most commonly resulted from a desire to clarify the role of a particular gene/enzyme in a tolerance mechanism.

4.1 Yeasts

Perhaps the most extensively studied ethanologenic organism is *Saccharomyces cerevisiae*. The foundations of traditional brewing of wines, beer, and sake are built around this and related organisms and their intrinsic ability to ferment various sugars (mostly glucose and sucrose) to ethanol (Attfield et al. 1997). Ethanol tolerance aside, industrial yeast strains have to demonstrate the ability to tolerate a number of process stresses, such as high temperature, fluctuations in pH, and osmotic stress. The tolerance to ethanol beyond that which is intrinsic to the species (approximately 12–14% by volume for *S. cerevisiae*) is a desirable characteristic for an ethanol fuel bioprocess based on yeast.

Improved ethanol tolerance in yeasts has been attributed to a number of factors, including an upregulation of the general stress response, permanent beneficial plasma membrane compositional changes (Alexandre et al. 1994; Alexandre and Charpentier 1994), and improved capacity for accumulation of intracellular osomoprotectants, such as trehalose (Ogawa et al. 2000). Tolerance has also been shown to vary with process optimization, such as medium composition (Kadokura et al. 1996), temperature (Ciesarova et al. 1996), and osmotic influences (Ciesarova et al. 1996). Ethanol has been reported to impair mitochondrial function, as seen in the evolution of "petit" cells in a fermenting population. Rho-petits arise as a result of ethanol accumulation in industrial fermentations and are a result of mitochondrial membrane impairment. Ethanol-tolerant strains of *S. cerevisiae* typically display low incidences of Rho-petits and likewise have high ergosterol/phospholipid ratios within cellular membranes (Argueso et al. 2009; Attfield et al. 1997), thought to counteract the fluidizing effects of ethanol. Interestingly, it appears that

the ethanol tolerance was, in this case, a direct result of mitochondrial functional evolution. The transference of mitochondria from wine yeast strains with low susceptibility to the Rho-petit phenotype to laboratory strains also transferred ethanol resistance (Argueso et al. 2009; Attfield et al. 1997; Patnaik 2008).

The application of proteomics has produced a number of system-wide studies seeking to understand the global physiological response of strains to various stressors, such as ethanol, and to harness the data to improve and adapt strains to higher alcohol environments. In general, the various microarray, proteomic, and genomic studies support and expand the empirical observations that had formed the foundation of the physiological understanding of stress response (Patnaik 2008; Yoshikawa et al. 2009). Strategies to improve ethanol tolerance have included single gene overexpression/deletion studies, global transcription machinery engineering, which aims to alter the expression of a number of RNA polymerase II-dependent genes (Alper et al. 2006), and whole genome shuffling (Zhao and Bai 2009). Several studies have reported the presence of stress response elements (STRE) in the promoter sequences of a variety of *S. cerevisiae* genes, such as those encoding heat shock proteins (Watanabe et al. 2009). These elements bind transcription factors, such as Msn2p and Msn4p, which, in turn, activate the expression of genes which contain STRE in their promoter regions and thus impart tolerance to a variety of stresses to the strain (Ogawa et al. 2000; van Voorst et al. 2006). The overexpression of Msn2 in a *sake* yeast strain has been shown to impart improved ethanol tolerance (Watanabe et al. 2009), and the role of Msn and the STRE has now been well described in this and related strains.

Global transcription machinery engineering (gTME) is an approach that reprograms gene transcription for particular phenotypes; an example is the mutagenesis of the transcription factor Spt15p of *S. cerevisiae*, which increased tolerance to ethanol (Alper et al. 2006). Whole genome shuffling has yielded similar results through protoplast fusion and genome recombination of multiple parents that possess individual yet desirable characteristics (Shi et al. 2009; Wei et al. 2008). With these and other technologies, such as directed evolution and protein engineering, it is clear that further improvements can be made to strains in order to engineer the specific phenotypes required for industrial purposes.

4.2 Mesophilic Bacteria

Much of our understanding of the physiology of solvent production, tolerance, and associated resistance mechanisms comes from the study of relatively few isolates of the genus *Pseudomonas* (*P. putida* and *P. aeruginosa*) and from the workhorse of molecular biology and genetics, *E. coli*. In *Pseudomonas* species, the focus has mostly been on understanding the physiology of the solvent-tolerant phenotype. Gene deletion technology has been a primary tool to test the role of specific genes in a particular solvent resistance pathway. For example, it has recently been reported that the insertion of noncoding sequence into the *srp*S gene of *P. putida* S12, which

knocked out the SrpS repressor protein of the multidrug efflux pump SrpABC, effectively generated a strain constitutively expressing SrpABC and displaying an extreme solvent-tolerant phenotype (Sun and Dennis 2009).

The major current biotechnological focus is, however, on improving ethanol and butanol tolerance in production organisms, such as engineered variants of *E. coli* (Luo et al. 2009; Yomano et al. 1998; Zhou et al. 2008) and the butanol-producing members of the genus *Clostridium* (Alsaker et al. 2004; Borden and Papoutsakis 2007). Here the focus is more directed to the generation and characterization of solvent-tolerant strains under process conditions, such as when fermenting complex carbohydrate feedstocks. *E. coli* mutants with increased tolerance have been generated. These were found to be defective in the *marR* gene that encodes a repressor protein for the *mar* operon (responsible for environmental stress factors) (Aono 1998). In addition, increasing the expression of the *sox*S, *mar*A, and *rob*A stress-response genes, all of which encode DNA-binding proteins/transcriptional activators, has been shown to increase tolerance in several strains of *E. coli* through regulation of the AcrAB–TolC system (Aono 1998; Asako et al. 1999). More recently, a fatty acid desaturase from *Bacillus subtilis* (Des) and a b-hydroxydecanoyl thio-ester dehydrase from *E. coli* (*fab*A) were coexpressed in *E. coli*, resulting in a mutant strain with increased ethanol tolerance compared to the wild type (Luo et al. 2009). Of particular note in the field of improved ethanologenic *E. coli* variants is the development of ethanol-tolerant mutants of *E. coli* strain KO11, which contains the *pdc* and *adh*II genes from *Z. mobilis* (Yomano et al. 1998). Ethanol-tolerant *E. coli* variants of strain KO11, capable of producing more than 60 gL^{-1} ethanol from xylase, have been isolated, reportedly exceeding the tolerance threshold of their engineered *S. cerevisiae* and *Z. mobilis* counterparts (xylose-fermenting strains) (Yomano et al. 1998). One of these ethanol-tolerant variants was subsequently found to possess a nonfunctional *fnr* transcriptional activator gene (Gonzalez et al. 2003; Yomano et al. 1998). The mutants LY01, LY02, and LY03, when subjected to a 0.5-min exposure to 10% ethanol, showed a survival rate of over 50%, as compared to below 10% for the parental strain KO11 (Gonzalez et al. 2003; Yomano et al. 1998).

A number of ethanol-tolerant bacterial strains have emerged from the wine and brewing industries. For example, members of genus *Lactobacillus* are common contaminants of traditional brewing but have been isolated and characterized because of their ability to tolerate the high ethanol concentrations of wine fermentations. The work with *Oenococcus* strains has contributed to the understanding of membrane fluidity and dynamics under high-ethanol conditions (Da Silveira et al. 2003; Silveira et al. 2004; Tourdot-Marechal et al. 2000). For example, a total of 76 *Lactobacillus plantarum* and *Oenococcus oeni* strains were isolated from a red wine fermentation and were shown to grow in the presence of up to 13% ethanol at 18°C (Alegria et al. 2004). Subsequently, even more ethanol-tolerant variants have been engineered, such as strains of *Lactobacillus plantarum* strain WCFS1 that overproduce *hsp*18.5, *hsp*18.55, and *hsp*19.3 translationally fused to the start codon of the *ldh*L promoter (Fiocco et al. 2007). Viability, compared to the wild-type strain, was improved in the presence of 1% v/v butanol and 12% v/v ethanol.

It is apparent from surveys of the literature that microorganisms are generally much less butanol tolerant than ethanol tolerant (Zheng et al. 2009). Even in butanol-producing strains, butanol tolerance is not particularly high with the well-studied butanol producer, *C. acetobutylicum,* possessing poor tolerance to the solvent, typically around 1% v/v (Lee et al. 2008b; Zheng et al. 2009). Butanol-tolerant variants have been developed from wild-type strains of *C. acetobutylicum* using a variety of methods, such as classic chemical mutagenesis, continuous culture selection, and serial enrichment procedures (Lee et al. 2008a, b; Zheng et al. 2009). Butanol-tolerant mutants derived from these experiments include the *C. acetobutylicum* ATCC 824 mutant strains SA-1, SA-2, and G1 which have increases in long acyl chain fatty acids and increased membrane fluidity. However, in some cases, these mutants have lost the butanol production phenotype (Baer et al. 1987). Using a DNA microarray approach, it has been shown that in *C. acetobutylicum*, a variety of "stress" genes are upregulated in response to acetate, butyrate, and butanol stress. These include *dna*K, *gro*ES, *gro*EL, *hsp*90, and *hsp*18 (Alsaker et al. 2010).

These genes have become the targets of strain development strategies, such as the reported overexpression of the *gro*ESL operons in *C. acetobutylicum* ATCC 824 which resulted in a higher yielding and butanol-tolerant variant (Alsaker et al. 2010). It was also demonstrated that overexpression of *C. acetobutylicum* Spo0A (a transcriptional regulator of solvent synthesis genes) increased butanol tolerance. This effect was a component of an enhanced general stress response that included the upregulation of genes involved in DNA synthesis, cell division, glycolysis, and butanol synthesis and various heat shock proteins (Alsaker et al. 2010).

4.3 Thermophilic Bacteria

Because of significant interest in the discovery and development of second-generation biofuel processes designed around strains possessing broad catabolic ranges and enhanced alcohol production capacities, several research groups have sought new metabolically diverse and adaptable isolates from more extreme environments. There is particular focus on thermophilic species from the genera *Clostridium* (possessing the ability to degrade crystalline cellulose; Dien et al. 2003), *Thermoanaerobacter* (Georgieva and Ahring 2007; Georgieva et al. 2007a, b, 2008; Klinke et al. 2001), and *Geobacillus* (Cripps et al. 2009). These organisms are all catabolically promiscuous. Their thermophilic characteristics potentially impart an additional process advantage: the facilitated removal of volatile product at high temperatures by application of a gas stream or under vacuum (Ezeji et al. 2004, 2005). The value of facilitating downstream product removal could also circumvent issues of low alcohol tolerance in the production strain and could be exploited in the development of a commercial process. However, work has also focused on adapting thermophilic bacteria to survive higher concentrations of solvent (Taylor et al. 2009).

In an early attempt to develop an ethanol-tolerant strain of *C. thermocellum* (the wild-type strains in this genus are typically tolerant to approximately 4% v/v

ethanol), it was concluded that adaptive responses included control of the alcohol production rate, yield, and concentration (Herrero and Gomez 1980). Subsequently, it was reported that an increase in the short unsaturated and anteisobranched fatty acid content was found to be associated with ethanol shock, resulting in increased membrane "fluidity" in *C. thermocellum* (Demain et al. 2005; Herrero and Gomez 1980; Williams et al. 2007). Ethanol-tolerant mutants of *Clostridium thermohydrosulfuricum* (growing in up to 8% v/v ethanol) have been selected by serial passaging of actively growing cultures into media containing successively higher concentrations of ethanol (Lovitt et al. 1984). Such mutants have been described as possessing enhanced growth, increased tolerance to various solvents, broader catabolic substrate ranges, and differences in fermentation end product ratios (Lovitt et al. 1984, 1988). Ethanol tolerance was shown to be temperature-dependent in the mutant but not in the parent strain. It was demonstrated that in the parent strain, low ethanol tolerance was not a result of disruption of membrane fluidity or glycolytic enzyme activity (Lovitt et al. 1984, 1988).

A mutant strain of *Thermoanaerobacter ethanolicus* (39E H8) that was tolerant to 8% v/v ethanol, lacked the primary *adh* (associated with ethanol consumption), and increased the percentage of transmembrane fatty acids (long-chain C_{30} fatty acids) in response to the increased levels of ethanol generated from a secondary Adh enzyme (ethanol-producing) has been reported (Burdette et al. 2002). Consistent with observations that ethanol tolerance in thermophiles is temperature-dependent, *Thermoanaerobacter* strain A10 was able to tolerate 4.7% v/v but was completely inhibited at 5.6% v/v ethanol when grown on xylose at 70°C. In comparison, ethanol tolerance was higher at lower temperatures (at 60°C, the strain could tolerate 5.1% v/v ethanol) (Georgieva et al. 2007b).

Species of the genus *Geobacillus* are generally tolerant to a maximum of 4% v/v ethanol (tolerance is defined here as the ability to actively grow; Taylor et al. 2009). Recently, however, two novel strains denoted as M5EXG and M10EXG, which were tolerant to 5% and 10% v/v ethanol, respectively, have been isolated from compost (Fong et al. 2006). Both strains were capable of fermenting arabinose, galactose, mannose, glucose, and xylose and produced low amounts of ethanol, potentially making them ideal candidates for development as ethanologenic strains (Fong et al. 2006; Tang et al. 2009). Such developments have already been initiated with strains of *G. thermoglucosidasius* NCBI 11955 (Cripps et al. 2009). Related species, such as *Anoxybacillus* sp. WP0, which was reported to grow optimally at 60°C and maintained viability in 15% v/v ethanol, are also potential candidates for further development (Peng et al. 2008b).

4.4 Microalgae and Cyanobacteria

The majority of microalgae are phototrophic microorganisms, i.e., they produce energy using photosynthesis to drive fixation of dissolved CO_2 as their major carbon source. The concept of using microalgae in biofuel production relies on using their

ability to capture and transform diffuse and irregular renewable energy (light and chemical energy) into algae biomass, a stable "high-energy" substrate. This substrate is a resource for the generation of extraction of secondary products. Many microalgae species are able to produce industrially relevant solvents, and their biofuel production yields are promising (Metzger and Largeau 2005; Xu et al. 2010).

For microalgae and cyanobacteria, solvent toxicity is based on the solvent's incorporation within membrane lipids causing disruption of essential cellular functions, enzyme inactivation, breakdown of transport mechanisms, and, at high concentrations, cell lysis. The solvents' toxicity is dependent on the nature of both the solvent and the microorganism (Okumura et al. 2001). The level of solvent tolerance of microalgae was found to be intermediate between that reported for bacteria and for plant cell suspensions (Leon et al. 2001). However, it should be noted that the green microalga *Botryococcus braunii* produces a wide range of hydrocarbons which can represent up to 61% of their dry weight (Metzger and Largeau 2005).

While the mechanisms of solvent tolerance in microalgae and cyanobacteria are not yet fully understood, some unusual and unique metabolic pathways which may be implicated in cellular tolerance exist. The microalga *Chlorella vulgaris* SDC1 has been shown to metabolize isopropanol and to excrete the primary aerobic breakdown product, acetone, into the extracellular medium (McEvoy et al. 2004). The marine cyanobacterium *Phormidium valderianum* BDU 30501 degrades phenol through the intracellular activities of polyphenol oxidase and laccase, with phenol inducing a concentration-dependent increase in cellular protein. The latter could be explained by the *de novo* synthesis of phenol-degrading enzymes and stress-related proteins (Shashirekha et al. 1997). The metabolism of phenanthrene by the marine *Agmenellum quadruplicatum* PR-6 shows more similarities to the detoxification reactions catalyzed by mammalian liver microsomes than to the catabolic reactions catalyzed by bacteria that utilize phenanthrene as a source of carbon and energy (Narro et al. 1992).

4.5 Microbial Communities and Consortia

The interactions between different microbial consortia in the environment are important in maintaining the ecosystem's functionality. Their activities induce biogeochemical transformations in natural, managed, and engineered ecosystems and are particularly relevant in contaminated systems. The converse is also true, where the introduction of solvents and other contaminants induces modifications in microbial community structure, often leading to the selection of resistant organisms and the disappearance of sensitive ones (Bordenave et al. 2007). In the presence of complex pollutant mixtures, effective biodegradation often involves a combination of numerous tolerant organisms belonging to a variety of taxa and functioning as a microbial community. The ability of the community to tolerate the toxic

components of the system and catabolize these components, and to detoxify the environment is in itself a mechanism of resistance.

The impact of solvents on microbial communities has mostly been studied for polycyclic aromatic hydrocarbons (PAH) as they are highly toxic, mutagenic, and carcinogenic and persist in the environment. Microbial hydrocarbon degradation and the enzymatic pathways involved have been recently reviewed (Peng et al. 2008c; Widdel and Rabus 2001). The greater ability of multiple species to resist solvents and toxic compounds than single species has been demonstrated experimentally. A consortium of two *Pseudomonas* spp. (*P. putida* and *P. alcaligenes*) and two *Acinetobacter* spp. (*A. baumannii* and *A. johnsonii*) were reported to be able to degrade and tolerate higher concentrations of phenol (up to 2,000 mg L^{-1}), with an increased specific consumption rate (0.71 g phenol g^{-1} cell h^{-1}) compared to the individual and even paired strains (Prpich and Daugulis 2005). The principle demonstrated by this example has mostly been reported in communities mediating the degradation of (chloro)phenolics (Prpich and Daugulis 2005; Schmidt et al. 1983), but has also been reported in environments contaminated with (iso)propanol (Bustard et al. 2000, 2001), BTX (benzene, toluene, xylene) (Oh and Bartha 1997), and phenanthrene (Munoz et al. 2003)

The addition of other solvents to an already contaminated system has also been shown to have effect on community tolerance dynamics. In a residual hydrocarbon-contaminated environment, the presence of added ethanol has been shown to enhance the community efficiency of hydrocarbon biodegradation (Feris et al. 2008; Lawrence et al. 2009; Parales et al. 2000). In such circumstances, up to 100-fold increases in the expression levels of the aerobic catabolic genes *dmpN* (phenol hydroxylase) and *todC1* (toluene dioxygenase) have been observed, together with the proliferation and selection of *Azospirillum* and *Brevundimonas* spp., which are known hydrocarbon degraders (Capiro et al. 2008).

Chemotaxis, which enables motile microorganisms to locate pollutants and is implicated in biofilm formation, surfactant pollution, and expression of specific genes (Parales et al. 2000), has been further implicated in community resistance dynamics. The formation of biofilms in particular can facilitate the degradation of solvents by bacterial consortia. Biofilms also provide a "protected" environment in which cultures can exist at higher than "normal" concentrations of solvent. This has been demonstrated with highly solvent-tolerant Gram-negative bacteria which successfully biofiltrate high concentrations of isopropanol (Bustard et al. 2001).

5 Applications for Biofuels

A strain capable of producing an economically valuable metabolite with a clear commercial value, such as an alcohol, has great value for biotechnological application. The current range of microbially produced compounds that have value as biofuels or biofuel additives is summarized in Fig. 2, which indicates where different feedstock derivatives can fuel production of various metabolites. The

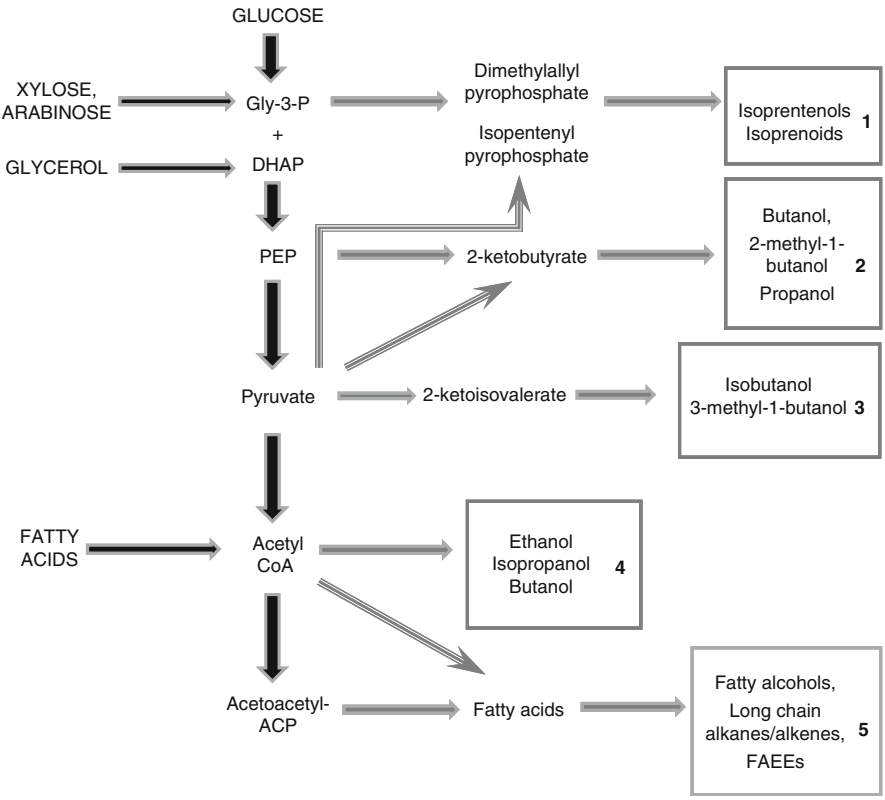

Fig. 2 A general schematic demonstrating the catabolic incorporation of "biofuel substrates" and potential products generated therefrom: (1) isoprenoid derivatives, (2–3) nonfermentative alcohols, (4) fermentative alcohols, and (5) fatty acid derivatives. Gly-3-P Glyceraldehyde-3-phosphate, DHAP dihydroxyacetone phosphate, PEP phosphoenolpyruvate, FAEEs fatty acid ethyl esters

development of such strains typically encompasses multiple phases and requires integration into a successful bioprocess (Papoutsakis 2008; van Zyl et al. 2007; Zheng et al. 2009). The development of commercial bioalcohol processes based on lignocellulosic feedstocks is an illustrative example. Lignocellulosic pretreatment and hydrolysis must result in a fermentable feedstock (a liquor) that contains carbohydrates suitable for fermentation by the process strain. The concentrations of toxic compounds, such as acetate and furfural, released during pretreatment need to be maintained at below inhibitory levels. The process organism is therefore required to be catabolically diverse, adaptable to fluctuations in pH and temperature, and capable of producing an alcohol at yields close to theoretical maximum. In addition, due to the toxic effects of alcohol accumulation, a more tolerant organism would have a positive impact on product yields and process costs.

The development of ethanol tolerance in a number of strains has resulted in improved product yield and process hardiness and therefore has impacted positively on the commercial viability of bioethanol production (Basso et al. 2008; Goldemberg 2006). Other approaches to achieving economically viable bioethanol production, such as commercial fermentations that operate at high temperatures with thermophilic hosts, have relied on the removal of the toxic ethanol from the locality of the organism. Ethanol-tolerant strains are still of interest despite this process circumvention (Taylor et al. 2009).

The difficulties in the commercialization of butanol fermentation bioprocesses lie in the severe toxicity of butanol at low concentrations and the higher boiling point of butanol, even beyond that which supports extremophilic life. Nevertheless, successful commercial ventures have been established in China and Russia (Chiao and Sun 2007; Nimcevic and Gapes 2000; Zverlov et al. 2006) where continuous culture and gradual adaptation have proved to be successful for selection of robust fermentation strains. Alternative strategies to minimize the impact of butanol toxicity include the continuous removal of butanol by pervaporation, liquid–liquid extraction, or gas stripping (Durre 2008; Ezeji et al. 2004; Lee 2008). Despite the current technical limitations of homobutanologenic fermentations, there is apparently considerable optimism that technical challenges can be resolved; it has recently been reported that butanol plants have been planned in many countries (Ezeji et al. 2010; Liu and Qureshi 2009; Nimcevic and Gapes 2000; Zheng et al. 2009)

Solvent tolerance is a characteristic that can also be effectively applied to processes other than biofuel production, in particular, in the field of biotransformation (Faizal et al. 2005; Nijkamp et al. 2007; Ramos-Gonzalez et al. 2003; Watanabe et al. 2008). For example, the highly solvent-tolerant *P. putida* DOT-T1E is an ideal candidate for the biotransformation of highly toxic substrates and has been engineered for biotransformation of toluene into the industrially relevant 4-hydroxybenzoate (4-HBA; Ramos-Gonzalez et al. 2003). 4-HBA is used in the synthesis of paraben and methylparaben, which are themselves used for the synthesis of liquid glass and are antimicrobial agents. The engineered *P. putida* strain T-57 (which has the ability to utilize *n*-butanol, toluene, styrene, *m*-xylene, ethylbenzene, *n*-hexane, and propylbenzene as growth substrates) is also able to catalyze the biotransformation of toluene into cresol in two-phase (organic-aqueous) systems (Faizal et al. 2005).

6 Conclusion and Perspectives

A number of themes which underpin the principles of solvent tolerance emerge from this review. The ability to alter cellular membrane composition and structure appears to be a widespread and critical response. This is generally achieved through the increased expression of saturated membrane fatty acids or modification of existing fatty acids via *cis–trans* isomerization. An alternative mechanism, well characterized in *Pseudomonas* spp., is the active exclusion of solvent molecules

from the cell via efflux pumps, such as AcrABC. The level of research in this area is now at a point where the crystal structures and mechanisms of action of the constituent proteins are available and described. For both these general resistance mechanisms, a greater understanding of their underlying regulatory systems is also emerging. These include theories on the regulation of *cti* and detailed characterization of the regulatory proteins of *acr*ABC and related operons. With the impact of "omic" technologies (in particular, proteomics, which has been the approach that has typically uncovered identification of enzymes/proteins involved in stress response networks in yeast and in many other strains studied), a broader understanding of physiological responses in a variety of solvent-tolerant strains has been achieved. The concept of a "general cell response" is now widely described as a key component of an organism's response to solvent exposure. At a higher level, the interactions within microbial communities that constitute collective solvent resistance characteristics are beginning to be unraveled.

The understanding and application of solvent tolerance are now ranging beyond those few genera (such as *Pseudomonas*) which have been intensively studied. A wide range of genera and engineered variants therein, including *Bacillus* (and related genera), *Thermoanaerobacter*, and *Clostridium*, are now described as of biotechnological relevance as a result of their solvent tolerance and/or processing capacities. The underlying theme is to develop even more solvent-tolerant strains for specific industrial applications. There is no doubt that research on both the understanding and improvement of microbial solvent tolerance will continue, leading to new native and recombinant strains and ultimately to new applications. Two obvious targets for the immediate future are the development of strains which are more tolerant to ethanol and/or butanol. The butanol-producing *Clostridia* spp. and engineered butanol-producing strains of *E. coli* are likely to be the principal foci of these studies. Such research is strongly driven by both commercial and political demands for a wider range of functional fuels and fuel additives. Beyond the remit of biofuel production, solvent tolerance has arisen as a major focus in a number of bioremediation and biotransformation processes, such as the treatment of wastewaters and contaminated soils and the bioretrieval of valuable compounds from pollutant soils/waters.

References

Aguilera F, Peinado RA, Millan C, Ortega JM, Mauricio JC (2006) Relationship between ethanol tolerance, H+−ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. Int J Food Microbiol 110:34–42

Alegria GE, Lopez I, Ruiz JI, Saenz J, Fernandez E, Zarazaga M, Dizy M, Torres C, Ruiz-Larrea F (2004) High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol Lett 230:53–61

Alexandre H, Charpentier C (1994) The plasma-membrane ATPase of *Kloeckera-apiculata*: purification, characterization and effect of ethanol on activity. World J Microbiol Biotechnol 10:704–708

Alexandre H, Berlot JP, Charpentier C (1994) Effect of ethanol on membrane fluidity of protoplasts from *Saccharomyces-cerevisiae* and *Kloeckera-apiculata* grown with or without ethanol, measured by fluorescence anisotropy. Biotechnol Tech 8:295–300

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568

Alsaker KV, Spitzer TR, Papoutsakis ET (2004) Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. J Bacteriol 186:1959–1971

Alsaker KV, Paredes C, Papoutsakis ET (2010) Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*. Biotechnol Bioeng 105:1131–1147

Alterthum F, Ingram LO (1989) Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Appl Environ Microbiol 55:1943–1948

Aono R (1998) Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. Extremophiles 2:239–248

Aono R, Tsukagoshi N, Yamamoto M (1998) Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12. J Bacteriol 180:938–944

Aono R, Kobayashi M, Nakajima H, Kobayashi H (1995) A close correlation between improvement of organic solvent tolerance levels and alteration of resistance toward low levels of multiple antibiotics in *Escherichia coli*. Biosci Biotechnol Biochem 59:213–218

Aono R, Negishi T, Aibe K, Inoue A, Horikoshi K (1994) Mapping of organic solvent tolerance gene ostA in *Escherichia coli* K-12. Biosci Biotechnol Biochem 58:1231–1235

Argueso JL, Carazzolle MF, Mieczkowski PA, Duarte FM, Netto OV, Missawa SK, Galzerani F, Costa GG, Vidal RO, Noronha MF, Dominska M, Andrietta MG, Andrietta SR, Cunha AF, Gomes LH, Tavares FC, Alcarde AR, Dietrich FS, McCusker JH, Petes TD, Pereira GA (2009) Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. Genome Res 19:2258–2270

Asako H, Kobayashi K, Aono R (1999) Organic solvent tolerance of *Escherichia coli* is independent of OmpF levels in the membrane. Appl Environ Microbiol 65:294–296

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJ, Hanai T, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 10:305–311

Attfield P, Myers D, Hazell B (1997) The importance of stress tolerance to baker's yeast. Australas Biotechnol 7:149–154

Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl Environ Microbiol 53:2854–2861

Basso LC, de Amorim HV, de Oliveira AJ, Lopes ML (2008) Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 8:1155–1163

Borden JR, Papoutsakis ET (2007) Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl Environ Microbiol 73:3061–3068

Bordenave S, Goni-Urriza MS, Caumette P, Duran R (2007) Effects of heavy fuel oil on the bacterial community structure of a pristine microbial mat. Appl Environ Microbiol 73:6089–6097

Bothast RJ, Nichols NN, Dien BS (1999) Fermentations with new recombinant organisms. Biotechnol Prog 15:867–875

Buchholz SE, Eveleigh DE (1990) Genetic modification of *Zymomonas mobilis*. Biotechnol Adv 8:547–581

Burchhardt G, Ingram LO (1992) Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. Appl Environ Microbiol 58:1128–1133

Burdette DS, Jung SH, Shen GJ, Hollingsworth RI, Zeikus JG (2002) Physiological function of alcohol dehydrogenases and long-chain (C-30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. Appl Environ Microbiol 68:1914–1918

Bustard MT, Meeyoo V, Wright PC (2001) Biodegradation of isopropanol in a three phase fixed bed bioreactor: start up and acclimation using a previously-enriched microbial culture. Environ Technol 22:1193–1201

Bustard MT, McEvoy EM, Goodwin JA, Burgess JG, Wright PC (2000) Biodegradation of propanol and isopropanol by a mixed microbial consortium. Appl Microbiol Biotechnol 54:424–431

Cann AF, Liao JC (2010) Pentanol isomer synthesis in engineered microorganisms. Appl Microbiol Biotechnol 85:893–899

Capiro NL, Da Silva ML, Stafford BP, Rixey WG, Alvarez PJ (2008) Microbial community response to a release of neat ethanol onto residual hydrocarbons in a pilot-scale aquifer tank. Environ Microbiol 10:2236–2244

Carman GM, Levin RE (1977) Partial purification and some properties of tyrosine phenol-lyase from *Aeromonas phenologenes* ATCC 29063. Appl Environ Microbiol 33:192–198

Chen TC, Levin RE (1975) Isolation of *Aeromonas* sp. ATCC 29063, a phenol-producing organism, from fresh haddock. Appl Microbiol 30:120–122

Chiao JS, Sun ZH (2007) History of the acetone-butanol-ethanol fermentation industry in China: development of continuous production technology. J Mol Microbiol Biotechnol 13:12–14

Choi HJ, Kim SA, Kim DW, Moon JY, Jeong YK, Joo WH (2008) Characterization of *Pseudomonas* sp. BCNU 171 tolerant to organic solvents. J Basic Microbiol 48:473–479

Ciesarova Z, Smogrovicova D, Domeny Z (1996) Enhancement of yeast ethanol tolerance by calcium and magnesium. Folia Microbiol 41:485–488

Clomburg JM, Gonzalez R (2010) Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology. Appl Microbiol Biotechnol 86:419–434

Cripps RE, Eley K, Leak DJ, Rudd B, Taylor M, Todd M, Boakes S, Martin S, Atkinson T (2009) Metabolic engineering of *Geobacillus thermoglucosidasius* for high yield ethanol production. Metab Eng 11:398–408

Da Silveira MG, Golovina EA, Hoekstra FA, Rombouts FM, Abee T (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. Appl Environ Microbiol 69:5826–5832

de Carvalho CC, da Cruz AA, Pons MN, Pinheiro HM, Cabral JM, da Fonseca MM, Ferreira BS, Fernandes P (2004) *Mycobacterium* sp., *Rhodococcus erythropolis*, and *Pseudomonas putida* behavior in the presence of organic solvents. Microsc Res Tech 64:215–222

Dellomonaco C, Fava F, Gonzalez R (2010) The path to next generation biofuels: successes and challenges in the era of synthetic biology. Microb Cell Fact 9:3

Demain AL, Newcomb M, Wu JH (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69:124–154

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266

Ding J, Huang X, Zhang L, Zhao N, Yang D, Zhang K (2009) Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 85:253–263

Doan TTP, Carlsson AS, Hamberg M, Bulow L, Stymne S, Olsson P (2009) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166:787–796

Durre P (2008) Fermentative butanol production: bulk chemical and biofuel. Ann N Y Acad Sci 1125:353–362

Essam T, Amin MA, El Tayeb O, Mattiasson B, Guieysse B (2010) Kinetics and metabolic versatility of highly tolerant phenol degrading *Alcaligenes* strain TW1. J Hazard Mater 173:783–788

Ezeji T, Milne C, Price ND, Blaschek HP (2010) Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. Appl Microbiol Biotechnol 85:1697–1712

Ezeji TC, Qureshi N, Blaschek HP (2004) Butanol fermentation research: upstream and downstream manipulations. Chem Rec 4:305–314

Ezeji TC, Qureshi N, Blaschek HP (2007a) Bioproduction of butanol from biomass: from genes to bioreactors. Curr Opin Biotechnol 18:220–227

Ezeji TC, Qureshi N, Blaschek HP (2007b) Production of acetone butanol (AB) from liquefied corn starch, a commercial substrate, using *Clostridium beijerinckii* coupled with product recovery by gas stripping. J Ind Microbiol Biotechnol 34:771–777

Ezeji TC, Karcher PM, Qureshi N, Blaschek HP (2005) Improving performance of a gas stripping-based recovery system to remove butanol from *Clostridium beijerinckii* fermentation. Bioprocess Biosyst Eng 27:207–214

Faizal I, Dozen K, Hong CS, Kuroda A, Takiguchi N, Ohtake H, Takeda K, Tsunekawa H, Kato J (2005) Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. J Ind Microbiol Biotechnol 32:542–547

Feris K, Mackay D, de Sieyes N, Chakraborty I, Einarson M, Hristova K, Scow K (2008) Effect of ethanol on microbial community structure and function during natural attenuation of benzene, toluene, and o-xylene in a sulfate-reducing aquifer. Environ Sci Technol 42:2289–2294

Fiocco D, Capozzi V, Goffin P, Hols P, Spano G (2007) Improved adaptation to heat, cold, and solvent tolerance in *Lactobacillus plantarum*. Appl Microbiol Biotechnol 77:909–915

Fischer CR, Klein-Marcuschamer D, Stephanopoulos G (2008) Selection and optimization of microbial hosts for biofuels production. Metab Eng 10:295–304

Fischer-Romero C, Tindall BJ, Juttner F (1996) *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. Int J Syst Bacteriol 46:183–188

Fong JC, Svenson CJ, Nakasugi K, Leong CT, Bowman JP, Chen B, Glenn DR, Neilan BA, Rogers PL (2006) Isolation and characterization of two novel ethanol-tolerant facultative-anaerobic thermophilic bacteria strains from waste compost. Extremophiles 10:363–372

Fralick JA (1996) Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J Bacteriol 178:5803–5805

Fujita K, Matsuyama A, Kobayashi Y, Iwahashi H (2006) The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols. FEMS Yeast Res 6:744–750

Fujita M, Hanaura Y, Amemura A (1995) Analysis of the rpoD gene encoding the principal sigma factor of *Pseudomonas putida*. Gene 167:93–98

Georgieva TI, Ahring BK (2007) Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. Appl Microbiol Biotechnol 77:61–68

Georgieva TI, Skiadas IV, Ahring BK (2007a) Effect of temperature on ethanol tolerance of a thermophilic anaerobic ethanol producer *Thermoanaerobacter* A10: modeling and simulation. Biotechnol Bioeng 98:1161–1170

Georgieva TI, Mikkelsen MJ, Ahring BK (2007b) High ethanol tolerance of the thermophilic anaerobic ethanol producer *Thermoanaerobacter* BG1L1. Cent Eur J Biol 2:364–377

Georgieva TI, Mikkelsen MJ, Ahring BK (2008) Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. Appl Biochem Biotechnol 145:99–110

Goldemberg J (2006) The ethanol program in Brazil. Environ Res Lett 1:1–10

Gonzalez R, Tao H, Purvis JE, York SW, Shanmugam KT, Ingram LO (2003) Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli:* comparison of KO11 (parent) to LY01 (resistant mutant). Biotechnol Prog 19:612–623

Heipieper HJ (2005) Adaptation of *Escherichia coli* to ethanol on the level of membrane fatty acid composition. Appl Environ Microbiol 71: 3388; author reply 3388

Heipieper HJ, Meinhardt F, Segura A (2003) The cis-trans isomerase of unsaturated fatty acids in *Pseudomonas* and *Vibrio:* biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiol Lett 229:1–7

Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74:961–973

Herrero AA, Gomez RF (1980) Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. Appl Environ Microbiol 40:571–577

Hild HM, Stuckey DC, Leak DJ (2003) Effect of nutrient limitation on product formation during continuous fermentation of xylose with *Thermoanaerobacter ethanolicus* JW200 Fe(7). Appl Microbiol Biotechnol 60:679–686

Ingram LO (1990) Ethanol tolerance in bacteria. Crit Rev Biotechnol 9:305–319

Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420–2425

Ingram LO, Gomez PF, Lai X, Moniruzzaman M, Wood BE, Yomano LP, York SW (1998) Metabolic engineering of bacteria for ethanol production. Biotechnol Bioeng 58:204–214

Inoue A, Yamamoto M, Horikoshi K (1991) *Pseudomonas putida* which can grow in the presence of toluene. Appl Environ Microbiol 57:1560–1562

Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H (2008) Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 77:1305–1316

Isken S, de Bont JA (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229–238

Junker F, Ramos JL (1999) Involvement of the cis/trans isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181:5693–5700

Kadokura T, Nakazato A, Takeda M, Chikubu S, Tanimura W (1996) Influence of culture liquid of fungi on alcohol tolerance of yeast. Seibutsu-Kogaku Kais 74:167–170

Keis S, Sullivan JT, Jones DT (2001a) Physical and genetic map of the *Clostridium saccharobutylicum* (formerly *Clostridium acetobutylicum*) NCP 262 chromosome. Microbiology 147:1909–1922

Keis S, Shaheen R, Jones DT (2001b) Emended descriptions of C*lostridium acetobutylicum* and *Clostridium beijerinckii,* and descriptions of *Clostridium saccharoperbutylacetonicum* sp nov and *Clostridium saccharobutylicum* sp nov. Int J Syst Evol Microbiol 51:2095–2103

Kim K, Lee S, Lee K, Lim D (1998) Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J Bacteriol 180:3692–3696

Kim Y, Ingram LO, Shanmugam KT (2007) Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. Appl Environ Microbiol 73:1766–1771

Klinke HB, Thomsen AB, Ahring BK (2001) Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by *Thermoanaerobacter mathranii*. Appl Microbiol Biotechnol 57:631–638

Koukkou AI, Tsoukatos D, Drainas C (1993) Effect of ethanol on the sterols of the fission yeast *Schizosaccharomyces-pombe*. FEMS Microbiol Lett 111:171–175

Lacis LS, Lawford HG (1992) Strain selection in carbon-limited chemostats affects reproducibility of *Thermoanaerobacter ethanolicus* fermentations. Appl Environ Microbiol 58:761–764

Lawrence A, Jonsson S, Borjesson G (2009) Ethanol, BTEX and microbial community interactions in the E-blend contaminated soil slurry. Int Biodeter Biodegrad 63:654–666

Lee KY (2008) Viscosity of high-alcohol content fuel blends with water: Subsurface contaminant transport implications. J Hazard Mater 160:94–99
Lee SK, Chou H, Ham TS, Lee TS, Keasling JD (2008a) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr Opin Biotechnol 19:556–563
Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS (2008b) Fermentative butanol production by Clostridia. Biotechnol Bioeng 101:209–228
Leon R, Garbayo I, Hernandez R, Vigara J, Carlos Vilchez C (2001) Organic solvent toxicity in photoautotrophic unicellular microorganisms. Enzyme Microb Technol 29:173–180
Li XZ, Poole K (1999) Organic solvent-tolerant mutants of *Pseudomonas aeruginosa* display multiple antibiotic resistance. Can J Microbiol 45:18–22
Li XZ, Zhang L, Poole K (1998) Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J Bacteriol 180:2987–2991
Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
Lin YP, Zhang MQ, Chen BQ (2005) Research progress of ethanologenic *Zymomonas mobilis*. Wei Sheng Wu Xue Bao 45:472–477
Liu S, Qureshi N (2009) How microbes tolerate ethanol and butanol. N Biotechnol 26:117–121
Lovitt RW, Longin R, Zeikus JG (1984) Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 48:171–177
Lovitt RW, Shen GJ, Zeikus JG (1988) Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. J Bacteriol 170:2809–2815
Luo LH, Seo PS, Seo JW, Heo SY, Kim DH, Kim CH (2009) Improved ethanol tolerance in *Escherichia coli* by changing the cellular fatty acids composition through genetic manipulation. Biotechnol Lett 31:1867–1871
Lynd LR, Grethlein HE, Wolkin RH (1989) Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. Appl Environ Microbiol 55:3131–3139
Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE (2008) How biotech can transform biofuels. Nat Biotechnol 26:169–172
McEvoy E, Wright PC, Bustard MT (2004) The effect of high concentration isopropanol on the growth of a solvent tolerant strain of *Chlorella vulgaris*. Enzyme Microb Technol 35:140–146
Metzger P, Largeau C (2005) *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl Microbiol Biotechnol 66:486–496
Mishra P, Kaur S (1991) Lipids as modulators of ethanol tolerance in yeast 34. Appl Microbiol Biotechnol 34:697–702
Mohagheghi A, Evans K, Chou YC, Zhang M (2002) Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of *Zymomonas mobilis* AX101. Appl Biochem Biotechnol 98:885–898
Mosqueda G, Ramos JL (2000) A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the tod genes for toluene metabolism. J Bacteriol 182:937–943
Mosqueda G, Ramos-Gonzalez MI, Ramos JL (1999) Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. Gene 232:69–76
Munoz R, Guieysse B, Mattiasson B (2003) Phenanthrene biodegradation by an algal-bacterial consortium in two-phase partitioning bioreactors. Appl Microbiol Biotechnol 61:261–267
Narro ML, Cerniglia CE, Van Baalen C, Gibson DT (1992) Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl Environ Microbiol 58:1351–1359

Ni M, Leung DYC, Leung MKH (2007) A review on reforming bio-ethanol for hydrogen production. Int J Hydrogen Energy 32:3238–3247

Nijkamp K, Westerhof RG, Ballerstedt H, de Bont JA, Wery J (2007) Optimization of the solvent-tolerant *Pseudomonas putida* S12 as host for the production of p-coumarate from glucose. Appl Microbiol Biotechnol 74:617–624

Nimcevic D, Gapes JR (2000) The acetone-butanol fermentation in pilot plant and pre-industrial scale. J Mol Microbiol Biotechnol 2:15–20

Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shimoi H, Ito K (2000) Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J Biosci Bioeng 90:313–320

Oh YS, Bartha R (1997) Construction of a bacterial consortium for the biofiltration of benzene, toluene and xylene emissions. World J Microbiol Biotechnol 13:627–632

Okumura Y, Koyama J, Takaku H, Satoh H (2001) Influence of organic solvents on the growth of marine microalgae. Arch Environ Contam Toxicol 41:123–128

Papoutsakis ET (2008) Engineering solventogenic clostridia. Curr Opin Biotechnol 19:420–429

Parales RE, Ditty JL, Harwood CS (2000) Toluene-degrading bacteria are chemotactic towards the environmental pollutants benzene, toluene, and trichloroethylene. Appl Environ Microbiol 66:4098–4104

Patnaik R (2008) Engineering complex phenotypes in industrial strains. Biotechnol Prog 24:38–47

Peng H, Wu G, Shao W (2008a) The aldehyde/alcohol dehydrogenase (AdhE) in relation to the ethanol formation in *Thermoanaerobacter ethanolicus* JW200. Anaerobe 14:125–127

Peng H, Gao Y, Xiao Y (2008b) The high ethanol tolerance in a thermophilic bacterium *Anoxybacillus* sp. WP06. Sheng Wu Gong Cheng Xue Bao 24:1117–1120

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008c) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32:927–955

Pinkart HC, White DC (1997) Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. J Bacteriol 179:4219–4226

Prpich GP, Daugulis AJ (2005) Enhanced biodegradation of phenol by a microbial consortium in a solid-liquid two phase partitioning bioreactor. Biodegradation 16:329–339

Ramos JL, Duque E, Huertas MJ, Haidour A (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J Bacteriol 177:3911–3916

Ramos JL, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323–3329

Ramos JL, Duque E, Rodriguez-Herva JJ, Godoy P, Haidour A, Reyes F, Fernandez-Barrero A (1997) Mechanisms for solvent tolerance in bacteria. J Biol Chem 272:3887–3890

Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56:743–768

Ramos-Gonzalez MI, Ben-Bassat A, Campos MJ, Ramos JL (2003) Genetic engineering of a highly solvent-tolerant *Pseudomonas putida* strain for biotransformation of toluene to p-hydroxybenzoate. Appl Environ Microbiol 69:5120–5127

Rao SC, Jones LP (2004) Formation of higher alcohols and phenol by strains of *Zymomonas* sp. Acta Biotechnol 7:209–219

Rodriguez-Herva JJ, Garcia V, Hurtado A, Segura A, Ramos JL (2007) The ttgGHI solvent efflux pump operon of *Pseudomonas putida* DOT-T1E is located on a large self-transmissible plasmid. Environ Microbiol 9:1550–1561

Rogers PL, Jeon YJ, Lee KJ, Lawford HG (2007) *Zymomonas mobilis* for fuel ethanol and higher value products. Adv Biochem Eng Biotechnol 108:263–288

Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL, Segura A (2001) Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J Bacteriol 183:3967–3973

San Martin R, Bushell D, Leak DJ, Hartley BS (1992) Development of a synthetic medium for continuous anaerobic growth and ethanol production with a lactate dehydrogenase mutant of *Bacillus stearothermophilus*. J Gen Microbiol 138:987–996

Sardessai Y, Bhosle S (2002) Tolerance of bacteria to organic solvents. Res Microbiol 153:263–268

Schmidt E, Hellwig M, Knackmuss HJ (1983) Degradation of chlorophenol by a defined microbial community. Appl Environ Microbiol 46:1038–1044

Segura A, Duque E, Rojas A, Godoy P, Delgado A, Hurtado A, Cronan J, Ramos JL (2004) Fatty acid biosynthesis is involved in solvent tolerance in *Pseudomonas putida* DOT-T1E. Environ Microbiol 6:416–423

Shashirekha S, Uma L, Subramanian G (1997) Phenol degradation by the marine cyanobacterium *Phormidium vaderianum* BDU 30501. J Ind Microbiol Biotechnol 19:130–133

Shen CR, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng 10:312–320

Shi DJ, Wang CL, Wang KM (2009) Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 36:139–147

Sikkema J, de Bont JA, Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem 269:8022–8028

Sikkema J, de Bont JA, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201–222

Silveira MG, Baumgartner M, Rombouts FM, Abee T (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl Environ Microbiol 70:2748–2755

Sun X, Dennis JJ (2009) A novel insertion sequence derepresses efflux pump expression and preadapts *Pseudomonas putida* S12 for extreme solvent stress. J Bacteriol 191:6773–6777

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11

Tang YJ, Sapra R, Joyner D, Hazen TC, Myers S, Reichmuth D, Blanch H, Keasling JD (2009) Analysis of metabolic pathways and fluxes in a newly discovered thermophilic and ethanol-tolerant *Geobacillus* strain. Biotechnol Bioeng 102:1377–1386

Taylor MP, Eley KL, Martin S, Tuffin MI, Burton SG, Cowan DA (2009) Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends Biotechnol 27:398–405

Tourdot-Marechal R, Gaboriau D, Beney L, Divies C (2000) Membrane fluidity of stressed cells of *Oenococcus oeni*. Int J Food Microbiol 55:269–273

Touze T, Eswaran J, Bokma E, Koronakis E, Hughes C, Koronakis V (2004) Interactions underlying assembly of the *Escherichia coli* AcrAB-TolC multidrug efflux system. Mol Microbiol 53:697–706

Tran HTM, Cheirsilp B, Hodgson B, Umsakul K (2010) Potential use of *Bacillus subtilis* in a co-culture with *Clostridium acetobutylicum* for acetone-butanol-ethanol production from cassava starch. Biochem Eng J 48:260–267

Trinh CT, Unrean P, Srienc F (2008) A minimal *Escherichia coli* cell for most efficient ethanol production from hexoses and pentoses. Appl Environ Microbiol 74:3634–3643

van Voorst F, Houghton-Larsen J, Jonson L, Kielland-Brandt MC, Brandt A (2006) Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress. Yeast 23:351–359

van Zyl WH, Lynd LR, den Haan R, McBride JE (2007) Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 108:205–235

Waege I, Schmid G, Thumann S, Thomm M, Hausner W (2010) Shuttle vector-based transformation system for *Pyrococcus furiosus*. Appl Environ Microbiol 76:3308–3313

Wang Z, Chen M, Xu Y, Li S, Lu W, Ping S, Zhang W, Lin M (2008) An ethanol-tolerant recombinant *Escherichia coli* expressing *Zymomonas mobilis pdc* and *adh*B genes for enhanced ethanol production from xylose. Biotechnol Lett 30:657–663

Wang X, Wang S, Li W, Li Y, Zhang Y (2009) Tolerant mechanims of bacteria to organic solvents. Sheng Wu Gong Cheng Xue Bao. 25:641–649

Watanabe H, Tanji Y, Unno H, Hori K (2008) Rapid conversion of toluene by an acinetobacter sp. Tol 5 mutant showing monolayer adsorption to water-oil interface. J Biosci Bioeng 106:226–230

Watanabe M, Watanabe D, Akao T, Shimoi H (2009) Overexpression of MSN2 in a sake yeast strain promotes ethanol tolerance and increases ethanol production in sake brewing. J Biosci Bioeng 107:516–518

Wei P, Li Z, He P, Lin Y, Jiang N (2008) Genome shuffling in the ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. Biotechnol Appl Biochem 49:113–120

White DG, Goldman JD, Demple B, Levy SB (1997) Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in *Escherichia coli*. J Bacteriol 179:6122–6126

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259–276

Wiegel J, Ljungdahl LG, Rawson JR (1979) Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. J Bacteriol 139:800–810

Wierckx NJ, Ballerstedt H, de Bont JA, Wery J (2005) Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. Appl Environ Microbiol 71:8221–8227

Wierckx NJ, Ballerstedt H, de Bont JA, de Winde JH, Ruijssenaars HJ, Wery J (2008) Transcriptome analysis of a phenol-producing *Pseudomonas putida* S12 construct: genetic and physiological basis for improved production. J Bacteriol 190:2822–2830

Williams TI, Combs JC, Lynn BC, Strobel HJ (2007) Proteomic profile changes in membranes of ethanol-tolerant *Clostridium thermocellum*. Appl Microbiol Biotechnol 74:422–432

Xu Y, Isom L, Hanna MA (2010) Adding value to carbon dioxide from ethanol fermentations. Bioresour Technol 101:3311–3319

Yamamoto S, Kasai H, Arnold DL, Jackson RW, Vivian A, Harayama S (2000) Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. Microbiology 146(Pt 10):2385–2394

Yanase H, Nozaki K, Okamoto K (2005) Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis*. Biotechnol Lett 27:259–263

Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 20:132–138

Yoshikawa K, Tanaka T, Furusawa C, Nagahisa K, Hirasawa T, Shimizu H (2009) Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in *Saccharomyces cerevisiae*. FEMS Yeast Res 9:32–44

You KM, Rosenfield CL, Knipple DC (2003) Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl Environ Microbiol 69:1499–1503

Zahir Z, Seed KD, Dennis JJ (2006) Isolation and characterization of novel organic solvent-tolerant bacteria. Extremophiles 10:129–138

Zgurskaya HI, Nikaido H (1999a) Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. Proc Natl Acad Sci USA 96:7190–7195

Zgurskaya HI, Nikaido H (1999b) AcrA is a highly asymmetric protein capable of spanning the periplasm. J Mol Biol 285:409–420

Zgurskaya HI, Yamada Y, Tikhonova EB, Ge Q, Krishnamoorthy G (2009) Structural and functional diversity of bacterial membrane fusion proteins. Biochim Biophys Acta 1794:794–807

Zhang K, Sawaya MR, Eisenberg DS, Liao JC (2008) Expanding metabolism for biosynthesis of nonnatural alcohols. Proc Natl Acad Sci USA 105:20653–20658

Zhao XQ, Bai FW (2009) Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J Biotechnol 144:23–30

Zheng YN, Li LZ, Xian M, Ma YJ, Yang JM, Xu X, He DZ (2009) Problems with the microbial production of butanol. J Ind Microbiol Biotechnol 36:1127–1138

Zhou S, Iverson AG, Grayburn WS (2008) Engineering a native homoethanol pathway in *Escherichia coli* B for ethanol production. Biotechnol Lett 30:335–342

Zverlov VV, Berezina O, Velikodvorskaya GA, Schwarz WH (2006) Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. Appl Microbiol Biotechnol 71:587–597

Control of Stress Tolerance in Bacterial Host Organisms for Bioproduction of Fuels

Aindrila Mukhopadhyay, Nathan J. Hillson, and Jay D. Keasling

Contents

1 Introduction 210
2 Sources of Microbial Stress in Deconstructed Biomass 211
3 Targets for Engineering Stress Tolerance from Biomass Inhibitory Compounds 215
4 Impact of Engineering a Pathway 216
5 Accumulation of Toxic Products 218
5.1 Ethanol 219
5.2 Butanol 219
5.3 C5-10 Alcohols and Hydrocarbons 220
5.4 Targets for Engineering Stress Tolerance from Toxic End Products 221
6 Engineered Controls of Stress Tolerance Pathways 223
7 Robust Engineering of Multiple Tolerance Mechanisms into the Same Host 225
8 Conclusion and Perspectives 228
References 229

Abstract The need for renewable alternative sources of liquid biofuels has lead to tremendous interest in the conversion of lignocellulosic biomass to fuel compounds via microbial routes. A key aspect of the research involves the engineering of robust and stable microbial host platforms that can produce these compounds at high titer. Impact on growth caused by inhibitory compounds in the deconstructed biomass and accumulation of toxic metabolic intermediates and final product are bottlenecks that severely limit product titers. This chapter reviews known sources of toxicity arising from various aspects of this process and discusses native and heterologous mechanisms of microbial stress response and defense that can be used to engineer better production hosts.

A. Mukhopadhyay • N.J. Hillson • J.D. Keasling (✉)
Joint BioEnergy Institute, One Cyclotron Road, Building 978, Berkeley, CA 94720, USA
e-mail: keasling@berkeley.edu

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_9,

1 Introduction

Microorganisms have been engineered to produce an astonishingly large array of compounds ranging from high value pharmaceuticals, fragrances, and nutritional supplements to fine chemicals such as amino acids, solvents, and building blocks for paints, plastics, and polymers (Wackett 2008; Fortman et al. 2008; Klein-Marcuschamer et al. 2007). While the majority of research focuses on the development of optimal biosynthetic enzymes and pathways to convert selected carbon sources to target end products, the recent focus of microbial metabolic engineering on the production of bulk commodities, specifically biofuels and compounds otherwise derived from petrochemical sources (Burk 2010; Ryder 2009; Steen et al. 2010; Atsumi et al. 2008), has imposed the staggering additional challenge of maximizing production. Pursuing sustainable, ecologically friendly bio-routes remains important, but a key metric of success in the microbial production of biofuel compounds is reaching high production levels at minimal cost to compete with inexpensive petrochemical and synthetic methods. Several analyses have emphasized this overarching requirement of high biofuel production levels (Hill et al. 2006). For example, while typical production levels of n-butanol with clostridium strains are about 13 g/L, optimization to increase production to 19 g/L was required to make this process economically viable (Papoutsakis 2008). With ever-higher levels of production and the use of minimally processed biomass, other aspects of microbial cellular physiology become acutely significant (Zhang et al. 2009). Growth inhibitory factors from deconstructed lignocellulosic biomass, as well as the accumulation of toxic intermediates in the biosynthetic pathway and the final product itself, can limit production. Cellular engineering efforts must therefore shift to developing microbes that cope with growth inhibition, toxicity, and stress. Studies of microbial stress response toward these inhibitory factors are key to elucidating the mechanisms that may be utilized to generate a robust industrial host that can cope with all aspects of growth and production inhibition. Furthermore, microbial diversity, both in the form of the ever-growing repository of sequenced genomes as well as bio-prospecting new ecosystems, contains an immense potential to provide the mechanisms required to tolerate a range of inhibitory aspects presented by this biofuel production pipeline. This chapter describes commonly encountered inhibitors and toxic factors generated during the conversion of lignocellulosic material to biofuel, the corresponding mechanisms that can be brought to bear on stress mitigation, and the strategies to overcome current limitations in obtaining stable, engineered hosts for industrial use. While applicable to all microbial hosts used for large-scale production of compounds from deconstructed biomass, including *S. cerevisiae*, this chapter focuses on bacterial systems as the production host.

2 Sources of Microbial Stress in Deconstructed Biomass

Lignocellulosic biomass presents the most promising renewable source of feed for the production of liquid biofuels (Ragauskas et al. 2006). Plant biomass is largely comprised of cellulose, hemicellulose and lignin (Somerville et al. 2004). While it would be ideal to use all components of this available material, the present goal of biofuel programs is to maximize the use of sugar polymers, cellulose, and hemicellulose. Despite the focus on these sugar polymers, most downstream biological processes (saccharification and microbial conversion) cannot utilize this material directly. The main factors that contribute to the intractability of lignocellulosic material are the inaccessibility of cellulose in its crystalline form and the occlusion of hemicellulose and cellulose by lignin (Himmel et al. 2007; Simmons et al. 2008). Pretreatment of plant biomass is therefore necessary to simplify the lignocellulosic material prior to saccharification and microbial conversion. Methods for deconstructing plant biomass include dilute acid hydrolysis, ammonia fiber expansion, and most recently, the use of ionic liquids. All deconstruction methodologies generate by-products that are detrimental to microbial growth and/or impact the bioconversion of sugars to biofuels (Fig. 1).

Dilute acid pretreatment is the most widely utilized and best documented method for plant biomass deconstruction and is known to generate inhibitory by-products that fall into three main categories: (1) furan aldehydes (furfural and hydroxymethylfurfural (HMF)) formed via the degradation of xylose and glucose, respectively (Klinke et al. 2004; Palmqvist and Hahn-Hagerdal 2000a; Pienkos and Zhang 2009); (2) organic acids, namely acetic acid produced by the deacetylation of hemicellulose and lignin, formic and levulinic acids from furans and HMF, respectively, and gluconic acid (Himmel et al. 2007; Palmqvist and Hahn-Hagerdal 2000a); and (3) phenolic compounds and other aromatics from lignin breakdown (Palmqvist and Hahn-Hagerdal 2000b; Pienkos and Zhang 2009). Detailed studies have also identified a range of aromatic compounds, aldehydes, ketones, and other acids (Klinke et al. 2004; Ranatunga et al. 1997).

Ammonia fiber expansion (AFEX) is an alternate strategy to the dilute acid treatment. AFEX minimizes the formation of sugar degradation products and converts a greater portion of the cellulose to sugars (Wyman et al. 2009; Lau and Dale 2009) compared to the dilute acid procedure, though the latter may be more efficient for biomass with high woody content (Sun and Cheng 2002). Common inhibitors associated with AFEX are the phenolics derived from depolymerized lignin and their associated aromatic degradation products (Balan et al. 2009).

Ionic liquid-based pretreatment of cellulose, though suggested as early as 1934 (Swatloski et al. 2002), is a relatively new procedure for deconstructing lignocellulosic material (Li et al. 2009; Swatloski et al. 2002) and provides an alternative to dilute acid processing and AFEX (Liu et al. 2010; Li et al. 2009; Singh et al. 2009). As the most recent technology to be explored in this context, studies are still ongoing that will elucidate the composition of the deconstructed plant material derived from ionic liquid pretreatment. The pros and cons of water-immiscible

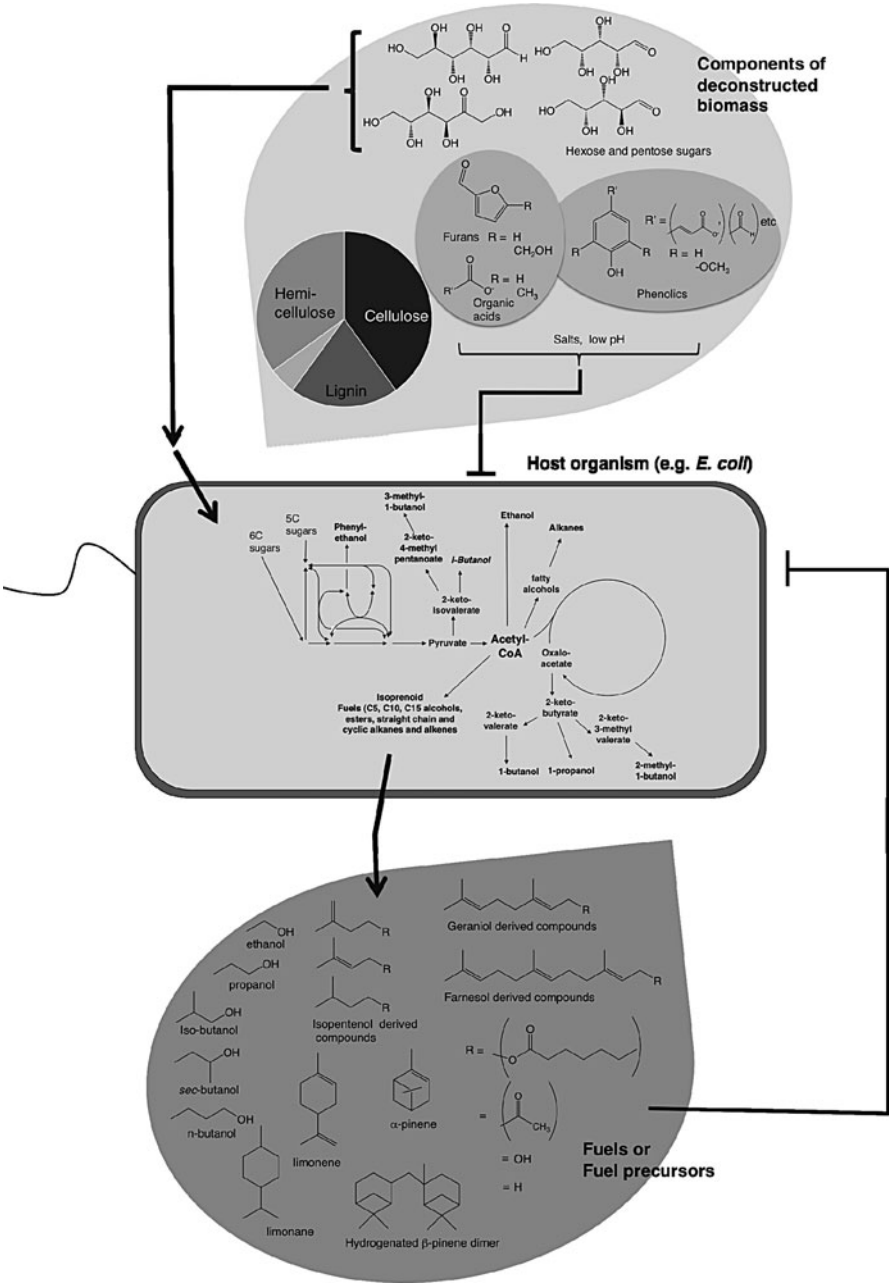

Fig. 1 Sources of common inhibitory compounds and toxic products. Sugar components of lignocellulosic plant biomass hydrolysates serve as carbon sources, while other components can have inhibitory impacts. A gram-negative bacterial host (e.g., *E. coli*) serves as the general host organism model with central metabolic routes leading to various classes of biofuels. Candidate biofuel compounds with known microbial toxicity are shown below. Note: the toxicities of *sec*-butanol, limonane, and the hydrogenated β-pinene dimer in *E. coli* have yet to be tested

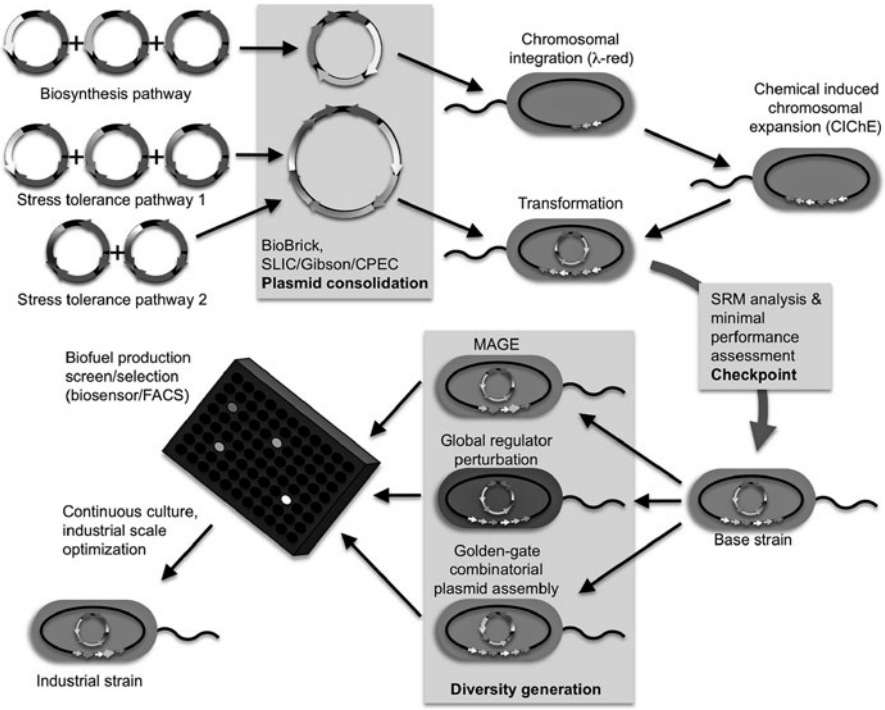

Fig. 2 Plasmid consolidation, CIChE, diversity generation, and strain selection process workflow. Biosynthesis and stress tolerance pathways are condensed into single plasmids using BioBrick (Anderson et al. 2010; Shetty et al. 2008), or SLIC/Gibson/CPEC (Gibson et al. 2009; Li and Elledge 2007; Quan and Tian 2009) methodologies. The biosynthetic pathway is then integrated into the chromosome, using the λ-red system, and subsequently expanded in the chromosome via CIChE (Tyo et al. 2009). The plasmid bearing the stress tolerance pathways is then transformed into the resulting strain. SRM analysis and performance assessments are conducted for each biosynthetic and stress tolerance pathway, ensuring that each pathway is at least minimally functional before proceeding to subsequent diversity generation with MAGE (Wang et al. 2009) (targeting the chromosomally expanded biosynthetic pathway and potentially other chromosomal loci), global regulator perturbation (Alper and Stephanopoulos 2007; Alper et al. 2006), and Golden-gate combinatorial plasmid assembly (Engler et al. 2008, 2009). Candidate strains are then screened or selected using a high-throughput assay such as a biofuel production biosensor (Dietrich and Keasling, unpublished data). Selected strains must then be optimized

ionic liquids have only recently begun to be explored (Park and Kazlauskas 2003; Li et al. 2009). Specifically, the impact of any residual levels of this reagent in the deconstructed soup on downstream processes, such as the saccharification steps or the microbial culture, requires to be studied.

Furan compounds (Fig. 1) have been thoroughly investigated for their impact on several bacterial hosts, such as *Zymomonas mobilis* (Ranatunga et al. 1997; Franden et al. 2009), several *E. coli* strains (Gutierrez et al. 2002, 2006; Zaldivar et al. 1999) including the ethanologenic *E. coli* LY180 (Miller et al. 2009a), and the solventogenic *Clostridium beijerinckii* (Ezeji et al. 2007). Very well studied in

S. cerevisiae (Horvath et al. 2001; Liu et al. 2005; Banerjee and Bhatnagar 1981; Gorsich et al. 2006), the toxicity is mitigated by aldehyde reductases that reduce the aldehydes to their corresponding furan methanols (Liu et al. 2004, 2008; Liu and Moon 2009; Petersson et al. 2006).

Lignin depolymerization yields a diverse array of phenolic alcohols, including coumaryl (no methoxy groups at the position *ortho-* to the OH- group), coniferyl (one methoxy group), and synapyl (two methoxy groups) (Fig. 1). Klinke et al. (2004) provide an extensive review of the toxicities of various alcohol, carbonyl and acid derivatives of these phenolic compounds, as well as their relative toxicities based on the number of methoxy groups. Ferulic acid and vanillin are among the best-studied phenolic compounds. Vanillin, in particular, has been used as an antimicrobial agent in the food industry (Fitzgerald et al. 2004; Gasson et al. 1998). The primary mechanism of phenolic toxicity universally appears to be the disruption of cell wall integrity.

Though not as toxic as the furan aldehydes or aromatic compounds, acetic acid is typically released in significant quantities and has been shown to impact not only growth but also target compound production. The latter has been documented for *Z. mobilis*, where the impact on ethanol production was greater than that explained by the impact on growth alone (Osman and Ingram 1985). Several studies have evaluated the effect of weak organic acids on bacterial physiology (Polen et al. 2003; Arnold et al. 2001). Acetic acid toxicity mainly arises from the membrane permeability of the undissociated acid. Upon entry into the cell, the acid dissociates and increases intracellular H^+ levels, decreasing the transmembrane proton gradient and disrupting the energy balance that is regulated by the proton motive force (Axe and Bailey 1995). Among other organic acids, accumulation of formic acid is another potential source of toxicity and is reported to elicit a very different general response from that of acetate accumulation (Kirkpatrick et al. 2001). Formic acid is reported to be more toxic than acetic acid (Pienkos and Zhang 2009) but typically accumulates at much lower levels during the pretreatment process. As such, most strain improvement efforts for small organic acids were focused on acetic acid (Dien et al. 2003; Pienkos and Zhang 2009; Warnecke and Gill 2005).

The most extensive studies examining the impact of a complete deconstructed soup on microbial host growth and production have focused on *S. cerevisiae* (Palmqvist and Hahn-Hagerdal 2000b) and implicate weak acids, phenolics and furans. Broad groups of inhibitory compounds and biomass hydrolysates have also been evaluated with ethanologenic *E. coli* (Klinke et al. 2004), *Z. mobilis* (Franden et al. 2009), and solventogenic clostridia (Mitchell et al. 2008). Similar toxic responses were identified in other bacterial hosts and methods to detoxify the deconstruction soup are often necessary. For example, overliming is a commonly used process for dilute acid pretreated material that has been shown to degrade many aromatic acids and ketones, (Klinke et al. 2004; Palmqvist and Hahn-Hagerdal 2000a) resulting in the production of gypsum and adding to the process cost (Galbe and Zacchi 2002). Cho et al. (2009) specifically targeted the peroxide-based removal of *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, vanillic acid, syringaldehyde, and vanillin, and demonstrated a clear improvement in

butanol production using *C. berjenkii*. While such removal methods have the potential to improve microbial conversion yields, they also add cost to the workflow. Therefore, it is worth examining microbial engineering of more resistant production strains so that residual amounts of these molecules do not impose any substantial impact on the microbial host.

3 Targets for Engineering Stress Tolerance from Biomass Inhibitory Compounds

The studies outlined in the previous sections provide a basis for cellular engineering for improved tolerance to the classes of inhibitory compounds discussed above. Dehydrogenases that convert furan aldehydes to less harmful alcohols have been documented in a wide variety of microbes, including *S. cerevisiae*, *P. putida*, and *E. coli*. However, despite their ability to metabolize HMF, these strains remain sensitive to the compound. The *E. coli* strain EMFR9, derived from the ethanologenic *E. coli* strain LY180, showed greater tolerance to furan aldehydes (Miller et al. 2009a). Analysis of this strain, under exposure to HMF, indicated that the genes (*yqhD* and *dkgA*) encoding two NADPH-dependent alcohol dehydrogenases that catalyze the conversion of furfural to furan methanol were repressed (Miller et al. 2009b). Although furan methanol is less toxic than furfural, the additional draw on NADPH impacted processes that use this cofactor, such as sulfur assimilation, and lead to a greater growth impact. Therefore, even though HMF detoxification pathways exist, the cofactors being utilized in the process should be kept in mind. In this regard, a recent study of an inhibitor tolerant *S. cerevisiae* strain found up-regulation in mechanisms that may offset the cofactor requirement for furfural and HMF reduction (Liu et al. 2009). Alternately, in situ detoxification strategies have also been explored that involve treating deconstructed biomass with strains that contain degradation pathways for aldehyde inhibitors prior to use with the fuel production host (Koopman et al. 2010; Wierckx et al. 2010). For discussions on molecular mechanisms of in situ detoxification of the aldehyde inhibitors in yeast, see Chap. 1.

In the case of phenolic compounds, several potential mechanisms exist that may alleviate or provide resistance to these inhibitory compounds. Efflux pumps that export inhibitory molecules provide a direct tolerance mechanism. Homologs of the aromatic acid efflux (Aae) pump system from *E. coli* (Van Dyk et al. 2004) and the toluene tolerance (Ttg) pumps in *P. putida* (Ramos et al. 2002) are potential candidates for the export of phenolic compounds. Modulation of the cell wall fatty acid composition has also been documented to provide benefit in coping with the disruptive action of phenolic compounds in *E. coli* (Keweloh et al. 1991). The metabolism or degradation of phenolic compounds to non-toxic metabolites is another potential route to mitigate phenolic stress. For example, phenol peroxidases (laccases) have been used to treat processed biomass (Jönsson

et al. 1998), and laccases from heterologous sources, such as the *Bacillus licheniformis* laccase *cotA*, have been functionally expressed in *E. coli* (Koschorreck et al. 2009). Other degradation mechanisms include decarboxylation, such as from ferulic acid to vinylguaiacol, a less toxic compound, which has been demonstrated in *Bacillus pumilus* (Lee et al. 1998). Interestingly, there are pathways to convert phenolic compounds to central metabolic intermediates, such as to acetyl-CoA via catechol, that are well documented in bacteria such as *Pseudomonas* spp. (Feist and Hegeman 1969; Ng et al. 1994; Herrmann et al. 1995). The meta-pathway that converts phenols to catechol and finally to acetyl-CoA consists of seven steps. Though it may be an elaborate route to obtain resistance, it provides the additional benefit of converting the inhibitory material to a central metabolism intermediate that can be channeled into cellular growth and production. This could be developed to maximize the use of all components of the lignocellulosic biomass, rather than just the sugar polymers.

With respect to small organic acids, long-term adaptation of *E. coli* to acetate has been undertaken and involved changes in metabolism (Holms and Bennett 1971; Polen et al. 2003) and is impacted by the choice of sugars in the carbon source (Lasko et al. 2000). Tolerance to these compounds has been studied and engineered in several host microbes (Dien et al. 2003; Pienkos and Zhang 2009; Warnecke and Gill 2005). See Chap. 5 for mechanisms of cell defense and tolerance to organic acid in yeast.

4 Impact of Engineering a Pathway

Commercially viable titers for bio-products can range from several mg/L in the case of pharmaceuticals to hundreds of g/L for commodity chemicals such as biofuels and are the primary driving force behind most metabolic engineering efforts. Reaching these production levels requires a significant amount of pathway optimization. Strain development is an iterative process whereby pathway manipulation is followed by system-level studies to identify potential bottlenecks and reveal detrimental side effects (Mukhopadhyay et al. 2008). Once it has been successfully demonstrated that a product of interest can be produced in vivo, achieving economically viable production levels requires minimizing the generation of less desirable side products and maximizing carbon flux toward the target product. For example, improvements in bio-ethanol production in *E. coli* has utilized many such steps, and this progress has been very well reviewed (Jarboe et al. 2007).

Most metabolic engineering efforts use a combination of native and heterologous genes. Examples include the production of the sesquiterpene amorphadiene (Newman et al. 2006; Martin et al. 2003), 1,3-propanediol (Saxena et al. 2009; Sauer et al. 2008; Biebl et al. 1999), 1,4-butanediol (Burgard and Van Dien 2007), iso-butanol (Connor et al. 2010; Cann and Liao 2008), and most recently, fatty acid ethyl ester production in *E. coli* (Steen et al. 2010). Understanding how the incorporation of an engineered exogenous pathway perturbs the host system is

important for overcoming pathway bottlenecks. For example, codon optimization of heterologous genes may be required to minimize stress caused by the depletion of the pool of available charged tRNA (Gustafsson et al. 2004; Welch et al. 2009). Additionally, the burden of expressing both native and non-native pathways can cause imbalances in the cellular redox state by altering the cofactor balance or levels of ATP, which can lead to overflow metabolism (Vemuri et al. 2006). Imbalances in enzymatic activity can also result in the accumulation of toxic or inhibitory pathway intermediates, which may drastically reduce cellular growth as well as production levels. A systematic evaluation in *E. coli* of intermediate buildup was conducted for an engineered isoprenoid pathway, converting the five carbon pyrophosphate intermediate to the final sequiterpene via the 10 and 15 carbon pyrophosphates, to examine the individual impact of each intermediate (Martin et al. 2003). The pyrophosphates were found to be highly detrimental to cellular growth in the order of C5 > C10 > C15, and a highly efficient final enzyme to convert the C15 farnesyl pyrophosphate to amorphadiene was required to relieve the system of stress. In another study, the accumulation of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) was found to be a bottleneck in the production of mevalonate via a heterologous pathway in *E. coli*. Downregulating the synthesis of HMG-CoA or the overexpression of *tHMG1,* encoding the enzyme downstream of HMG-CoA, alleviated the growth impact (Pfleger et al. 2006; Pitera et al. 2007).

Optimization of heterologous pathways is essential for maximizing production and minimizing the buildup of toxic intermediates. An elegant approach to alleviate HMG-CoA stress utilized scaffolding domains from metazoan signaling proteins to recruit the first three enzymes of the mevalonate pathway; all of which were tagged with the corresponding peptide ligands (Dueber et al. 2009). The scaffold co-localizes the enzymes thereby reducing the accumulation of toxic intermediates while increasing the effective concentrations of pathway intermediates in the vicinity of the enzymes. Varying the stoichiometry of the scaffold binding domains, effectively controlling enzyme ratios, resulted in 77-fold more mevalonate than the unscaffolded system.

In another instance, the host stress response was also a problem in the production of the *p*-hydroxy styrene precursor, *p*-hydroxy cinnamate, in *E. coli*. Systematic evaluation of the toxicity of *p*-hydroxy cinnamate led to discovery of the aromatic acid efflux genes (*aae*) (Van Dyk et al. 2004). Overexpression of the *aaeAB* genes using an inducible P_{trc} promoter resulted in a twofold increase in *p*-hydroxy cinnamate tolerance, while the toxicity from *p*-hydroxy styrene final product was alleviated using a biphasic reaction system (Sariaslani 2007; Van Dyk 2008). Recently optimization of *p*-hydroxy-styrene production using a solvent-resistant *P. putida* S12 strain also used an organic phase extraction system and improved production by twofold (Verhoef et al. 2009).

The accumulation of a particular intermediate does not necessarily indicate if it is due to excess levels of the upstream enzyme or the low levels of the downstream enzyme. Methods to compensate often express the limiting enzyme from a second plasmid and/or tune parameters such as the gene's promoter and ribosome binding site or the plasmid's origin of replication. However, arbitrary enzyme

overproduction can rob the cell of resources that could otherwise be devoted to generating the target compound. Tools that allow the quantitative interrogation of target enzymes and diagnostic methods that enable the evaluation of biosynthetic pathway expression provide key information for resolving pathway bottlenecks. High throughput mass spectrometric methods, such as selected reaction monitoring (SRM), are useful for diagnosing and optimizing protein production for biofuel production (Keasling 2008). Correlation of protein production levels with metabolite titers from different strains is integral to optimizing the productivity and stability of the engineered microbe (Dueber et al. 2009).

5 Accumulation of Toxic Products

The diversity of microbial biosynthetic pathways allows for a large number of biofuel candidates to be envisioned, but compounds must meet several criteria to serve as biofuel targets (Fig. 1). Several recent reviews comprehensively cover the range of microbially derived compounds that meet these criteria, ranging from small chain alcohols to alkanes and alkenes (for bio-gasoline) to longer chain hydrocarbons (for biodiesel) (Wackett 2008; Peralta-Yahya and Keasling 2010; Lee et al. 2008; Keasling and Chou 2008; Fortman et al. 2008; Chemier et al. 2009; Antoni et al. 2007) as well as cyclic hydrocarbons that may serve as bio-jet fuel components (Harvey et al. 2010; Ryder 2009).

Many of these compounds have solvent-like properties presenting a severe impact on cell growth and consequently limiting product titer. Even ethanol, the most well-established biofuel, is toxic at some level to the organisms used to produce it. Exposure to alcohols and solvents has been reported to impact bacterial growth via a variety of mechanisms including increased membrane fluidity, ion leakage, changes in fatty acid composition, difficulties in translation, and elongated cells (Baer et al. 1987; Ingram 1990; Sikkema et al. 1995; Tomas et al. 2004). In general, toxicity increases with solvent hydrophobicity, which is determined by the length of the carbon backbone. In general, the toxicity of the alcohol correlates well with the octanol–water partition coefficient, P_{ow}; at saturating concentrations, solvents with a log P_{ow} greater than 3.8 are not toxic to *E. coli*. The degree of toxicity of an alcohol varies across bacteria, with some bacteria being more affected by the length of the alkyl chain while others by saturation of the carbon backbone (fewer double bonds). The majority of toxicity studies propose the cell membrane as the most affected by organic solvents and as contributing significantly to stress adaptation. Short- and long-chain alcohols are known to cause stress by desiccation, and by intercalating into the hydrophobic cell wall fatty acids, respectively. Their similarities to other well-understood stresses, such as desiccation or hypersalinity, may suggest gene candidates for engineering fuel tolerant hosts. See Chap. 6 for more descriptions on microbial stress response to toxic compounds and organic solvent.

5.1 Ethanol

Microbial ethanol production from glucose and mixed sugars is now a well-established process. See Chap. 3 for molecular mechanisms of ethanol tolerance in yeast. A vast body of literature also focuses on bacterial ethanologenic hosts such as *E. coli* and *Z. mobilis* (Ingram et al. 1998; Jarboe et al. 2007; Lawford and Rousseau 2003; Lee 1997; Lin et al. 2005; Yomano et al. 1998; Zaldivar et al. 2001). The impact of various other parameters on ethanol production has also been investigated in *E. coli*. Examples include choice of sugar (Alterthum and Ingram 1989), acetic acid accumulation (Lawford and Rousseau 1992), inhibitors from lignocellulosic biomass (Zaldivar and Ingram 1999; Zaldivar et al. 1999, 2000), and loss of osmolytes (Underwood et al. 2004). *Z mobilis* is one of the best natural producers of ethanol and is naturally tolerant to greater amounts of ethanol than wild type *S. cerevisiae* or *E. coli* (Rogers et al. 1984), making it a focal point of many efforts for optimized ethanol production (Joachimsthal and Rogers 2000). *Z. mobilis*'s response to ethanol implicates heat shock response chaperones (Michel and Starka 1986; Barbosa et al. 1994), and early studies also found the lipid composition of *Z. mobilis* to be well suited for ethanol accumulation in having a large percentage of vaccenic acid in the acyl groups of its polar membrane phospholipids (Carey and Ingram 1983). However, even in *Z. mobilis*, the accumulation of ethanol eventually inhibits glucose uptake and conversion (Osman and Ingram 1985).

5.2 Butanol

Butanol stress has been extensively studied, for example, in solventogenic *Clostridia*, which is a native producer. In *C. acetobutylicum*, transcript analysis after exposure to 0.75% n-butanol (6 g/L) indicated that the primary response is the increase of transcripts encoding chaperones, proteases, and other heat shock-related proteins (Tomas et al. 2004), and further, the overexpression of GroELS chaperones produced strains with greater n-butanol tolerance (Tomas et al. 2003). To date, the most optimized butanol production in *C. acetobutylicum* leads to a titer of about 13.6 g/L while that in *C. berjenkii* is 19 g/L (Papoutsakis 2008). Several other clostridial strains are being investigated due to their ability to produce higher levels of n-butanol, such as *C. pasteurianum*, which can produce as high as 17 g/L using glycerol as a carbon source (Biebl 2001). Despite the availability of such natural producers, it has been argued that metabolic engineering of more tractable industrial hosts such as *E. coli* may be a better strategy for bio-butanol production. The main impediment toward this goal is the low concentration at which butanol is toxic to *E. coli*. Recent studies have examined the effect of n-butanol and iso-butanol exposure on *E. coli*. In the case of iso-butanol, transcript analysis revealed several key mechanisms including the disruption of quinone function and the involvement

of global regulators such as ArcA (Brynildsen and Liao 2009). n-Butanol stress in *E. coli* DH1 has also been examined in a comprehensive functional genomics study and was found to elicit strong cell envelope and oxidative stresses as well as cause perturbations to several ArcA-regulated electron transport and respiratory mechanisms (Rutherford et al. 2010).

The distribution of response among several major regulons makes it difficult to engineer all modes of stress relief, suggesting evolution and adaptation as important strategies to obtain stress-tolerant strains. Such non-targeted approaches were used in *Pseudomonas* spp, where n-butanol tolerance was improved from 3% to 6% (Ruhl et al. 2009). Nevertheless, the identification of genes impacted by n-butanol exposure in the functional genomics studies enables a systematic approach in which the corresponding knockdowns or overexpressions can be implemented and evaluated for improvement in solvent resistance. This targeted strategy has the distinct advantage of a well-defined approach that may be translated to other hosts. For example, n-butanol exposure caused a disruption of redox balance, points to candidates such as the alcohol dehydrogenase YqhD and superoxide dismutases (Rutherford et al. 2010).

5.3 C5-10 Alcohols and Hydrocarbons

An important class of biofuels can be derived from isoprenoid biosynthetic pathways. Hemi-, mono-, and sesquiterpenes (C5, C10, and C15, respectively) have all been suggested as potential fuel candidates. Specific examples include isopentenol, isopentanol (Connor and Liao 2009), limonene, limonane (from limonene (Ryder 2009)), dimethyl octane (from geraniol (Martin et al. 2007)); farnesane (from farnesene (Ryder 2009)), hydrogenated pinene dimers (Harvey et al. 2010); and others (Peralta-Yahya and Keasling 2010). Terpenes have historically been studied as medicinal, flavoring, and fragrance compounds. Limonene, pinene, geraniol and citronellol, putative biofuel compound precursors, are associated with plant extracts and are used in a wide array of cosmetics, insect repellant, sanitizing agents, and solvent applications. Toxicity of these isoprenoid compounds has been evaluated in a variety of bacteria such as *E. coli, Samonella enterica,* and *Staphylococcus aureus* (Kim et al. 1995; Trombetta et al. 2005; Cristani et al. 2007). Specific modes of antibiotic resistance have also been evaluated for several of these compounds. The common household disinfectant, Pinesol, contains a mixture of cyclic monoterpenes, and its antimicrobial impact on *E. coli* has been studied via transcript analysis (Gill et al. 2002). Following up on these initial studies, it was shown that the derepression of the AcrAB-TolC pump genes provided a significantly higher resistance to Pine oil (Moken et al. 1997). Similarly, oxidation-based mechanisms have been found in *Pseudomonas aeruginosa* for the metabolism of geraniol and citronellol (Hoschle and Jendrossek 2005).

Very few studies have evaluated the impact of longer carbon chain compounds on bacterial cultures as the solubility of these compounds drop below measurable

levels. It is likely that long-chain compounds beyond a certain carbon length no longer intercalate into the cell wall and will impose no toxic effect on cell growth or production. Consistent with this, no growth defect was observed for an *E. coli* strain developed to produce fatty acid ethyl esters (FAEE) at almost gram per liter scales (Steen et al. 2010). It is noteworthy, however, that addition of an organic phase to FAEE production cultures improved production by 1.6-fold (from 427 to 674 g/L), which may be suggestive of product accumulation causing a push back on the biosynthetic pathway.

5.4 Targets for Engineering Stress Tolerance from Toxic End Products

Targeted and systems-level studies in bacterial systems for solvent stress point to several candidates that may be explored to generate fuel-tolerant hosts. Selection of ethanol-tolerant *E. coli* is a much-explored area as is the application of cell-wide stress response studies and mutagenesis approaches (Ingram 1990; Jarboe et al. 2007; Jeffries and Jin 2000; Alper et al. 2006; Gonzalez et al. 2003; Yomano et al. 1998). Tolerance mechanisms range from modulation of cell wall fluidity (Ingram and Vreeland 1980; Ingram et al. 1980), expression of chaperones (Barbosa et al. 1994), to the use of osmoprotective agents such as glycine betain (Gonzalez et al. 2003). The response to ethanol is more like salt or desiccation stress in that ethanol appears to have a water exclusion effect. Consequently, studies implicate the role of osmoprotectants in stress mitigation (Gonzalez et al. 2003; Underwood et al. 2004). *E. coli* shows a similar response in cell wall fatty acid composition in response to salt and ethanol stresses and pretreatment with salt resulted in greater resistance to ethanol (Ingram and Vreeland 1980); specifically, an increase in unsaturated fatty acids was found in response to ethanol stress (Ingram et al. 1980). The opposite trends were observed during exposure to longer, more hydrophobic solvents such as hexenol, where pre-exposure to salt had no impact (Ingram et al. 1980). An increase in trans unsaturated fatty acids in response to both ethanol and NaCl was also found in *P. putida* (Loffeld and Keweloh 1996). A previous study found similar trends in *P. putida* exposed to toluene and ethanol: an increase in saturated fatty acids in cells exposed to toluene, but the reverse in cells exposed to ethanol, leading the authors to suggest that the reduction in saturation in ethanol is a cause rather than a response (Heipieper and de Bont 1994). Modulation of cell wall fluidity appears to be a key response in several other microbes such as *Oenococcus oeni* (Grandvalet et al. 2008; Silveira et al. 2004) and *Z. mobilis* (Carey and Ingram 1983; Michel and Starka 1986). Genetic engineering of cell wall fatty acid distribution has been used in several bacteria with measured impact on stress tolerance or sensitivity. Unsaturated fatty acids in the membrane of the cyanobacterium *Synechocystis* spp. were increased by deleting the desaturase genes *desA* and *desD* (Sakamoto and Murata 2002; Allakhverdiev et al. 1999) and resulted in an increased salt tolerance.

Similarly, a knockout in the *cis*/*trans* isomerase *cti* in *P. putida* DOR-T1E (Junker and Ramos 1999) resulted in an increased sensitivity to toluene. Finally, the cyclopropyl fatty acid synthase (*cfa*) from *O. oeni* has been used to complement a corresponding knockout in *E. coli* to restore ethanol sensitivity (Grandvalet et al. 2008).

Butanol is more hydrophobic than ethanol and does not cause the same type of water exclusion stress. However, butanol is toxic to *E. coli* and other bacteria at much lower concentrations. Being membrane permeable, butanol causes stress at both cell envelope as well as intracellular levels. Therefore, addressing the impacted cellular components, as discovered via cell wide studies, might provide appropriate stress relief. Mechanisms that correct the disrupted redox state of the cell, such as superoxide dismutases or dehydrogenases (e.g., *yqhD*), may be effective. Though these mechanisms have never been directly explored for relieving butanol stress, they have been effective in dealing with redox stress (Kang et al. 2007; Perez et al. 2008), which is also observed during n-butanol exposure in *E. coli* (Rutherford et al. 2010). Deletion of *ydhD* specifically reduced iso-butanol production in *E. coli* (Atsumi et al. 2009), consistent with its importance in stress from these target compounds. Cues from other bacteria, such as *C. acetobutyliticum*, include the overexpression of GroELS chaperones (Tomas et al. 2003).

A limited number of studies have evaluated the impact of longer chain alcohols, alkanes, alkenes, cyclic hydrocarbons, and aromatic compounds on bacteria. With respect to terpenoid compounds, studies in *E. coli* point to export pumps as a key mechanism to reduce toxicity. Given its wide substrate range, the AcrAB-TolC system native to *E. coli* holds the potential of providing tolerance toward several terpene compounds (Gill et al. 2002; Moken et al. 1997). Homologous pumps exist in other more solvent-resistant bacteria and are worthy of examination for engineering host resistance. With respect to solvent tolerance, a large body of knowledge comes from studies in *Pseudomonas* spp (Ramos et al. 2002). The involvement of efflux pumps is documented in *P. putida* DOT-T1E (Ramos et al. 1998), *P. putida* S12 (Kieboom et al. 1998a,b), *P. putida* MTB6 (Huertas et al. 2000), *P. putida* GM73 (Kim et al. 1998), and *P. putida* F1 (Phoenix et al. 2003) and is possibly the primary mechanism of solvent tolerance in these bacteria. Of special note is the versatile, solvent-resistant pump (*srp*) from *P. putida* S12, which was shown to be induced in response to a variety of relevant compounds such as C5-C9 alkanes (moderate induction) and C5-C8 alcohols (strong induction), as well as aromatic solvents (Kieboom et al. 1998b). The other key resistance mechanism reported in several *P. putida* strains is the increase in *cis* to *trans* isomerization of cell wall fatty acids, which modulates cell wall fluidity. Solvent tolerant *E. coli* strains have been reported to demonstrate resistance to cyclohexane at concentrations typically lethal to the parent *E. coli* strain (Aono and Kobayashi 1997). Subsequent analysis of these solvent-resistant strains found a decrease in cell wall hydrophobicity and specifically reported changes in the lipopolysaccharide content. *P. putida* strains have also been documented to use vesicles to sequester and export toxic metabolites (Kobayashi et al. 2000); however, this is neither a widely observed mechanism nor would it be straightforward to engineer into a

heterologous host. Alternate responses include metabolism of the offending alkane (Roling et al. 2002; van Beilen et al. 2001; Spormann and Widdel 2000); however, with respect to improving product titers, product catabolism is not an ideal strategy to alleviate the stress.

6 Engineered Controls of Stress Tolerance Pathways

Typical laboratory systems use carefully selected combinations of inducible promoters, plasmid copy numbers, ribosomal binding sites, and terminators to expressed genes and pathways (Smolke 2009). Such systems are invaluable for demonstrating feasibility for a biosynthetic pathway or stress response function. Furthermore, in the case of high value commodities where the impact on cell growth due to stress or the cost of maintaining plasmid-borne systems is completely offset by the value of the target compound, no further engineering may be necessary. In this regard, maximizing the amount of target compound per culture cycle is necessary to reduce the reliance on scale up alone. Therefore, the longer the host can perform optimally under production conditions, the greater the yield from a given quantity of starting material and correspondingly the cost associated with deconstruction per cycle of production. In such large-scale settings, especially in a continuous process, it becomes a significant hindrance to (1) maintain a plasmid using a selection marker and (2) provide a constant concentration of the external inducer. The elimination one or both may result in significant cost benefit to the process.

A basic strategy to bypass the cost and effort associated with the addition of the inducers would entail the use of constitutive promoters that provide a constant level of gene expression. However, stress response mechanisms may not be the ideal systems for functional expression under conditions where stress is not present. Even with the most benign mechanisms, such as the expression of chaperones, constitutive expression burdens the cell with excessive protein production. In most cases, however, expression of the stress response mechanism comes at an even higher cost. For example, overexpression of efflux pumps can be toxic to the cell due to overloading of the protein translocation machinery used to target proteins to the membrane (Wagner et al. 2007), and careful tuning of pump expression is required to avoid growth inhibition (Wagner et al. 2008). A better strategy, therefore, is to have expression systems that are regulated using cellular cues rather than an externally added inducer. There are at least two interesting approaches for an internally regulated system (Dunlop et al. 2010). One such system would be where sources of toxicity, specifically the inhibitory compounds or the accumulating harmful target compound, would be detected and used to trigger the expression of the appropriate mechanism. The other approach would use regulatory mechanisms that become active during the conditions imposed by the inhibitory compounds or the accumulating harmful target compound, and place the genes encoding the resistance mechanism under the control of these regulators.

To execute the former, sensory proteins that can sense the inhibitory compounds and effect downstream responses are required. Bacterial two-component systems, typically comprised of sensor histidine kinase (HK) and a response regulator (RR), are an ideal mechanism for such a strategy. In these systems, the histidine kinase functions to sense extra- and intracellular signals and triggers signal transduction via a phosphotransfer to the cognate RR that in its active phophorylated state regulates cellular response, often by gene induction (Stock et al. 2000; Galperin et al. 2001; Gao and Stock 2009). The biodiversity from sequenced organisms provides a variety of two-component systems (Mascher et al. 2006). These include systems that sense many relevant compounds discussed in this chapter or conditions associated with their presence. For example, phenolic and aromatic compounds serve as signals for the *Agrobacterium tumefaciens* VirA/VirG system (e.g., acetosyringone) (Lee et al. 1995) and the *P. putida* TodS/TodR (Toluene) (Lau et al. 1997) (Busch et al. 2007). Acidic pH is sensed by variety of sensors including the *E. coli* PhoQ (Bearson et al. 1997), the *A. tumefaciens* VirA (Gao and Lynn 2005), and the *Sinorhizobium* ActX (Tittabutr et al. 2006). Sensor kinases are also known for other pertinent signals or stress responses, such as hexose sugar sensing by the *E. coli* UhbP (Island and Kadner 1993; Wright and Kadner 2001; Wright et al. 2000), cell density or quorum sensing by the *E. coli* QseC (Sperandio et al. 2002), cell envelope stress by the *E. coli* CpxA, and redox stress by the *E. coli* ArcB (Iuchi et al. 1990; Malpica et al. 2004).

It should be pointed out that heterologous expression of a two-component system in *E. coli* may not be sufficient to accomplish signal sensing, transduction, and gene regulation, and the corresponding response regulator may also require native sigma factors etc. (Lohrke et al. 2001). However, this problem can be bypassed by using only the sensory domains of the appropriate two-component system as a fusion protein with native *E. coli* systems. Such fusion systems have been made successfully using the well-characterized EnvZ/OmpR two-component systems that natively control *E. coli's* response to changes in osmolarity (Cai and Inouye 2002; Kishii et al. 2007). Well-cited examples include fusion sensory HKs in which the periplasmic and transmembrane domains of chemoreceptor Tar (aspartate sensor) or Trg (ribose sensor) were fused with the catalytic core of EnvZ (Baumgartner et al. 1994; Utsumi et al. 1989). The resulting Taz1 and Trz1 proteins enabled response regulator activation to Asp or ribose rather than osmotic change. In another example, intracellular O_2 was sensed by a FixL-EnvZ fusion and used to induce *ompC-gfp* (Kumita et al. 2003). Factors to be taken into consideration in order to generate such chimeric sensor histidine kinases for microbial engineering have been described recently (Salis et al. 2009). The advantage of such an approach is that it will be highly specific to the inhibitor in question and will not be triggered by other conditions. The obvious drawback is that a suitable sensor may not be known (e.g., furfural). However, in such cases, signals that are less specific (e.g., change in pH) but that correlate with the presence of the toxic compound or growth stage can be used.

The second strategy uses cues pertinent to the stress response or a pertinent growth/metabolic condition to control the expression of stress mitigating

mechanisms. Systems biology studies provide a broad suite of differentially expressed genes for the conditions of interest. Done with the right controls, these data sets allow the identification of genes that change specifically in response to the stressor, in this case, the inhibitors and toxic final products. The regulatory system that controls the up- or downregulation of the specifically responding genes can then be used to control the expression of the selected stress response mechanism. Conditions that correlate with the stress or the growth mode corresponding to target compound production can be selected, and genes that are differentially modulated in response to these conditions provide potential targets for this approach. For example, candidates to drive key pathways would be those that correlate with sugar utilization (e.g., diauxic shift), cell density (e.g., via quorum sensing), and stationary phase promoters; all of which are an integral part of culturing conditions for target compound production.

In *E. coli*, the availability of gene libraries can also provide powerful strategies to identify ideal candidates for creating such control systems. An important resource in this regard is the library of fluorescent transcriptional reporters generated in *E. coli* K12, in which *gfp* (green fluorescent protein) has been placed under control of about 2,000 native *E. coli* promoters (Zaslaver et al. 2006). This library was used to assess promoter function for the glucose–lactose diauxic shift and could potentially be used to screen for relevant promiscuous or specific pumps for a wide variety of conditions pertinent to the biofuel production workflow.

7 Robust Engineering of Multiple Tolerance Mechanisms into the Same Host

As discussed above, it is possible to engineer several classes of stress tolerance mechanisms into various biofuel-producing hosts. Naturally, it is of more importance to approach a simultaneous incorporation of multiple tolerance characteristics and biofuel synthesis pathways into the same host. This approach assumes that each of the individual stress tolerance and biofuel synthesis pathways has (separately) been introduced into the host organism, assays have been developed to gauge the performance of each pathway, the function of each pathway has been verified in the host, and the associated expression control systems have been minimally optimized. With those assumptions satisfied, three major challenges arise when attempting to engineer multiple pathways into a single host: (1) optimizing the performance of a given biological pathway often adversely affects other pathways in the same cell, (2) simultaneous optimization of all the pathways is required, and (3) generating sufficient library diversity within the collective pathways (from which to screen or select) and maintaining pathway stability become more difficult with each additional pathway.

In stark contrast with the engineering ideal, biological pathways are generally far from orthogonal. All activities transpiring within the cell are coupled to a greater or

lesser extent with each other. Any given stress tolerance or biofuel synthesis pathway may affect the intracellular environment (available cellular resources, membrane structure, redox balance, pH, etc.) to the detriment of the other engineered pathways. For example, adding an AcrA/B-TolC efflux pump to relieve terpene/limonene toxicity would result in exporting tetracycline from the cell (Okusu et al. 1996), a side effect that would diminish the expression of a biosynthetic pathway placed under the control of the P_{tet} promoter. Thus, although a pathway may have previously been introduced into and optimized for the host organism, the performance of the pathway might greatly diminish after the introduction of the other pathways of interest.

Since the introduced pathways will likely perturb each other's performance, this naturally leads to the requirement to screen or select for the desired function of multiple pathways simultaneously. At the beginning of this process, it is important to identify any grossly under-performing pathway(s) (whether stress tolerance or biosynthetic) and only initiate a systems-wide combinatorial screen/selection once a minimal level of activity is achieved for every pathway. The effort at this stage should be modest, because each pathway has previously been demonstrated to be functional, but some serial re-optimization of pathways that perform extremely poorly in their new context may be required. The targeted SRM approach (Anderson and Hunter 2006) could prove invaluable at this point to determine a functional pathway's component ratios before placing it in a new context. If other stress tolerance and biosynthetic pathways dramatically perturb these component ratios, SRM analysis can be applied in an iterative fashion to improve the under-performing pathway(s).

When incorporating multiple pathways into a single host, there are potentially many different parameters to optimize for each gene (gene variant, promoter, RBS, copy number), and the aggregate parameters must be combinatorially assessed by each pathway's assay. However, generating a large and diverse combinatorial library to screen, or from which to select, for optimal systemic performance, is rather futile without comprehensive high-throughput assays. Some functional assays are higher throughput than others, and an immediate concern is that an assay whose throughput is acceptable for optimizing an individual pathway may not be feasible for use within the context of a combinatorial screen. Since tolerance pathways are generally assessed via growth rates under increasing titers of exogenously introduced stress, and are readily transferable to high-throughput screens or selections, the burden falls predominantly upon assaying the biosynthetic pathway. In addition, restraint should be applied against over-optimizing stress tolerance pathways to the detriment of biofuel production. It may be best to exclusively screen or select for biofuel production, because optimizing production will implicitly address any underlying stress tolerance limitations.

While a validated fluorescence-activated cell sorting (FACS)-based assay of biofuel production would be ideal, such as a recent finding of an intracellular n-butanol biosensor (Dietrich and Keasling, unpublished data), single cell assays may not be applicable in all situations, especially if the cellular export of the biofuel is limiting. A general caveat to screening or selecting for biofuel production in

batch mode at a small scale is that the results may not be particularly applicable to continuous culture at an industrial scale, and further strain and culture optimizations will likely be necessary (Burgard and Van Dien 2007). Finally, in addition to screening combinations of stress tolerance and biosynthetic pathways, it can also be very fruitful to perturb global regulators for improved performance (Alper and Stephanopoulos 2007; Alper et al. 2006).

Before any attempts have been made to incorporate multiple pathways into the same host organism, stress tolerance and biofuel synthesis pathways are generally introduced into the cell on one or more replicating plasmid vectors. Since the number of compatible origins of replication is limited, the pathways of interest, the genes for which may be distributed across multiple plasmids, must often be consolidated into one or a few vectors. The traditional approach of restriction enzyme/multiple cloning site plasmid construction impedes this process, as it becomes more difficult to find amenable restriction sites with each pathway added to a given plasmid, and increasingly likely to necessitate the introduction of silent point mutations to disrupt the undesirable recognition sites. Furthermore, the traditional implementation of this process will almost certainly vary for each new combination of stress tolerance and biofuel synthesis pathways (since new restriction sites will be selected and new point mutations must be introduced), and therefore, it will often be necessary to restart the process from scratch. An alternative approach is to employ a standardized assembly strategy, such as the BioBricks method (Shetty et al. 2008; Anderson et al. 2010), which easily allows for the concatenation of multiple pathways together, in any combination.

Even though each pathway has already been incorporated into the target host and minimally optimized, it will likely be necessary to do so again within the context of all of the other pathways. Consolidating multiple pathways into a single plasmid (increasing plasmid size) or transforming multiple plasmids into the same host can affect plasmid copy number. It will generally be required, then, to screen/select various different combinations as described above. It should be noted that combinatorial library creation is potentially at odds with the binary BioBrick assembly method, because the cumulative library size is limited by number of colonies pooled after each assembly step (only two sequences are assembled together at time). However, it is possible to generate combinatorial libraries using other methods (Li and Elledge 2007; Gibson et al. 2009; Quan and Tian 2009; Engler et al. 2008, 2009) that allow for concurrent multi-part assembly while maintaining BioBrick compatibility for downstream applications.

Even after consolidating all of the desired stress tolerance and biosynthetic pathways into a few plasmids and selecting/screening for optimal combinations thereof, there remain numerous drawbacks to plasmid systems. Plasmids are not often utilized in an industrial context, because enforcing antibiotic selection pressure is not cost-effective, introduces additional cellular stress, and potentially reduces biofuel production. In addition, some plasmids do not segregate in an ordered fashion (unlike the chromosome), and this segregational instability can result in plasmid loss and accelerate the spread of mutations through the plasmid population that curtails the biofuel pathway while allowing the plasmid to

propagate (Tyo et al. 2009). Note that this concern is generally only applicable to the biofuel synthesis pathway (which itself may be responsible for cell stress), since in the presence of the cell-stress, even in the absence of antibiotic selection pressure, the stability of stress tolerance pathways may not justify significant concern.

Chromosomal integration is an important route to stabilizing biosynthetic pathways. While replicating plasmids offers variable copy-numbers to chose from (e.g., pUC vs. pSC101 (Smolke 2009)), chromosomal integrations have historically been limited to a single copy. Chromosomal integrations with multiple copies have recently been demonstrated with the *c*hemical *i*nduced *ch*romosome *e*xpansion (CIChE) method (Tyo et al. 2009). Whereas it has been relatively facile to generate combinatorial plasmid libraries (with variable promoters, RBS, etc.) from which to screen or select, it has been more challenging to accomplish the analogous chromosomal modifications. To some extent, with the advent of *m*ultiplex *a*utomated *g*enomic *e*ngineering (MAGE) (Wang et al. 2009), it is becoming feasible to achieve combinatorial diversity within the chromosome itself.

Recombination is yet another means available to the host organism to disable biosynthetic pathways. Cellular recombination machinery can remove (from the chromosome or a plasmid) portions of a deleterious pathway that contains high-homology sequence repeats (e.g., a repeated promoter sequence). In addition, since the aforementioned CIChE methodology relies upon only one repeat flanking the pathway to be integrated (Tyo et al. 2009), it is likely that the CIChE process will not result in chromosomal repeats of the entire pathway if the pathway internally contains repeated sequences. Perhaps the best defense against undesired pathway recombination is to avoid sequence repeats altogether, utilizing multiple gene operons and the minimal number of promoters and terminators, where possible. When multiple promoters and terminators with similar function are required, it is advisable to choose those with maximally divergent sequences. An additional means to mitigate pathway recombination instability is to delete *recA*, as performed at the completion of CIChE (and before initiating the MAGE process).

8 Conclusion and Perspectives

A biofuel-producing host must harbor not only the biosynthetic pathway, but also the carefully engineered tolerance mechanisms to enable stable growth and high production. Serious consideration of a bacterial host for the production of a bulk commodity must address issues of pretreatment inhibitors, metabolic engineering burden and toxicity from target compound. End product toxicity especially is a common problem in strain engineering for biotechnology applications and is possibly the most critical in biofuel production due the absolute requirement to maximize production titer. *E. coli* engineered to serve as an industrial host to produce bulk chemicals such as 1,4-butanediol and 1,3-propanediol, had substantial engineering devoted to improving tolerance was required to make production cost

effective (Burk 2010; Zeng and Biebl 2010). While the toxicity from some of current and advanced biofuel candidates are well understood, entire classes of fuels that can be produced microbially remain to be explored further. For example recent discoveries of novel pathways enabled the production of hydrocarbons longer than C15 in *E. coli* (Beller et al. 2010), and further developments will reveal if such targets impose toxic limits on the production titer and the strain engineering required to overcome these.

Eventually, the goals of a well-engineered microbial host system go beyond a typical laboratory inducer controlled, plasmid borne system. Beyond just the deployment of key tolerance mechanisms, not only will the target genes be expressed using cues from the system, but they will also have more sophisticated positive and negative feedback controls like those found in native microbial systems. Such engineered systems would allow more optimal levels of stress response to be maintained in the face of fluctuating stress conditions and variable product formation, potentially resulting in a more robust producer (Dunlop et al. 2010). Strategies for developing resistant marker free strains with chromosomally encoded pathways and tolerance mechanisms are also essential both to generate a stable host platform and also for ease and safety of use in large industrial scales.

In this chapter, we sought to identify common sources of cellular growth and toxicities that might be encountered by a biofuel producer and discuss several targeted approaches that may help in the development of a better producer. However, other combinatorial and evolutionary strategies also exist to address similar problems and are a well-reviewed topic (Zhang et al. 2009). Recent studies promise new strategies that can be brought to bear on strain engineering such as the genome scale technologies developed for compiling and transplanting a complete mycoplasma genome into a heterologous host (Gibson et al. 2010). Alternate genome level approaches are the use of metagenomic fosmids to engineer tolerance to pretreatment inhibitors (Sommer et al. 2010). The importance of cellular engineering to optimize microbial physiology beyond pathway optimization is being recognized as an important aspect of strain development especially in the conversion of lignocellulosic biomass to biofuels.

Acknowledgment This work conducted by the Joint BioEnergy Institute was supported by the Office of Science, Office of Biological and Environmental Research, of the US Department of Energy under Contract No. DE-AC02-05CH11231.

References

Allakhverdiev SI, Nishiyama Y, Suzuki I, Tasaka Y, Murata N (1999) Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of Synechocystis to salt stress. Proc Natl Acad Sci USA 96:5862–5867

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568

Alper H, Stephanopoulos G (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab Eng 9:258–267

Alterthum F, Ingram LO (1989) Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Appl Environ Microbiol 55:1943–1948

Anderson JC, Dueber JE, Leguia M, Wu GC, Goler JA, Arkin AP, Keasling JD (2010) BglBricks: a flexible standard for biological part assembly. J Biol Eng 4:1

Anderson L, Hunter CL (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 5:573–588

Antoni D, Zverlov VV, Schwarz WH (2007) Biofuels from microbes. Appl Microbiol Biotechnol 77:23–35

Aono R, Kobayashi H (1997) Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. Appl Environ Microbiol 63:3637–3642

Arnold CN, McElhanon J, Lee A, Leonhart R, Siegele DA (2001) Global analysis of *Escherichia coli* gene expression during the acetate-induced acid tolerance response. J Bacteriol 183:2178–2186

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJ, Hanai T, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 10:305–311

Atsumi S, Wu TY, Eckl EM, Hawkins SD, Buelter T, Liao JC (2009) Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. Appl Microbiol Biotechnol 85:651–657

Axe DD, Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol Bioeng 47:8–19

Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl Environ Microbiol 53:2854–2861

Balan V, Sousa LD, Chundawat SPS, Marshall D, Sharma LN, Chambliss CK, Dale BE (2009) Enzymatic digestibility and pretreatment degradation products of AFEX-treated hardwoods (*Populus nigra*). Biotechnol Prog 25:365–375

Banerjee N, Bhatnagar RLV (1981) Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. Eur J Appl Microbiol Biotechnol 11:226–228

Barbosa MF, Yomano LP, Ingram LO (1994) Cloning, sequencing and expression of stress genes from the ethanol-producing bacterium *Zymomonas mobilis*: the *groESL* operon. Gene 148:51–57

Baumgartner JW, Kim C, Brissette RE, Inouye M, Park C, Hazelbauer GL (1994) Transmembrane signalling by a hybrid protein: communication from the domain of chemoreceptor Trg that recognizes sugar-binding proteins to the kinase/phosphatase domain of osmosensor EnvZ. J Bacteriol 176:1157–1163

Bearson S, Bearson B, Foster JW (1997) Acid stress responses in enterobacteria. FEMS Microbiol Lett 147:173–180

Beller HR, Goh EB, Keasling JD (2010) Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*. Appl Environ Microbiol 76:1212–1223

Biebl H (2001) Fermentation of glycerol by *Clostridium pasteurianum*-batch and continuous culture studies. J Ind Microbiol Biotechnol 27:18–26

Biebl H, Menzel K, Zeng AP, Deckwer WD (1999) Microbial production of 1,3-propanediol. Appl Microbiol Biotechnol 52:289–297

Brynildsen MP, Liao JC (2009) An integrated network approach identifies the isobutanol response network of *Escherichia coli*. Mol Syst Biol 5:277

Burgard AP, Van Dien SJ (2007) Methods and organisms for the growth-coupled production of succinate. US Patent 2007/0111294 A1

Burgard AP, Van Dien SJ (2008) Methods and organisms for the growth-coupled production of succinate. Patent EP1937821A2

Burk MJ (2010) Sustainable production of industrial chemicals from sugars. Int Sugar J 112:30–35

Busch A, Lacal J, Martos A, Ramos JL, Krell T (2007) Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals. Proc Natl Acad Sci USA 104:13774–13779

Cai SJ, Inouye M (2002) EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. J Biol Chem 277:24155–24161

Cann AF, Liao JC (2008) Production of 2-methyl-1-butanol in engineered *Escherichia coli*. Appl Microbiol Biotechnol 81:89–98

Carey VC, Ingram LO (1983) Lipid composition of *Zymomonas mobilis*: effects of ethanol and glucose. J Bacteriol 154:1291–1300

Chemier JA, Fowler ZL, Koffas MA, Leonard E (2009) Trends in microbial synthesis of natural products and biofuels. Adv Enzymol Relat Areas Mol Biol 76:151–217

Cho DH, Lee YJ, Um Y, Sang BI, Kim YH (2009) Detoxification of model phenolic compounds in lignocellulosic hydrolysates with peroxidase for butanol production from *Clostridium beijerinckii*. Appl Microbiol Biotechnol 83:1035–1043

Connor MR, Cann AF, Liao JC (2010) 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. Appl Microbiol Biotechnol 86:1155–1164

Connor MR, Liao JC (2009) Microbial production of advanced transportation fuels in non-natural hosts. Curr Opin Biotechnol 20:307–315

Cristani M, D'Arrigo M, Mandalari G, Castelli F, Sarpietro MG, Micieli D, Venuti V, Bisignano G, Saija A, Trombetta D (2007) Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. J Agric Food Chem 55:6300–6308

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266

Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KL, Keasling JD (2009) Synthetic protein scaffolds provide modular control over metabolic flux. Nat Biotechnol 27:753–759

Dunlop MJ, Keasling JD, Mukhopadhyay A (2010) A model for improving microbial biofuel production using a synthetic feedback loop. Syst Synth Biol 4:95–104

Engler C, Gruetzner R, Kandzia R, Marillonnet S (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS ONE 4:e5553

Engler C, Kandzia R, Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS ONE 3:e3647

Ezeji T, Qureshi N, Blaschek HP (2007) Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. Biotechnol Bioeng 97:1460–1469

Feist CF, Hegeman GD (1969) Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J Bacteriol 100:869–877

Fitzgerald DJ, Stratford M, Gasson MJ, Ueckert J, Bos A, Narbad A (2004) Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. J Appl Microbiol 97:104–113

Fortman JLCS, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD (2008) Biofuel alternatives to ethanol: pumping the microbial well. Trends Biotechnol 26:375–381

Franden MA, Pienkos PT, Zhang M (2009) Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of *Zymomonas mobilis*. J Biotechnol 144:259–267

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618–628

Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol Lett 203:11–21

Gao R, Lynn DG (2005) Environmental pH sensing: resolving the VirA/VirG two-component system inputs for Agrobacterium pathogenesis. J Bacteriol 187:2182–2189

Gao R, Stock AM (2009) Biological insights from structures of two-component proteins. Annu Rev Microbiol 63:133–154

Gasson MJ, Kitamura Y, McLauchlan WR, Narbad A, Parr AJ, Parsons EL, Payne J, Rhodes MJ, Walton NJ (1998) Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA

hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J Biol Chem 273:4163–4170

Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, Benders GA, Montague MG, Ma L, Moodie MM, Merryman C, Vashee S, Krishnakumar R, Assad-Garcia N, Andrews-Pfannkoch C, Denisova EA, Young L, Qi ZQ, Segall-Shapiro TH, Calvey CH, Parmar PP, Hutchison CA III, Smith HO, Venter JC (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. Science 329:52–56

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA III, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345

Gill RT, Wildt S, Yang YT, Ziesman S, Stephanopoulos G (2002) Genome-wide screening for trait conferring genes using DNA microarrays. Proc Natl Acad Sci USA 99:7033–7038

Gonzalez R, Tao H, Purvis JE, York SW, Shanmugam KT, Ingram LO (2003) Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli*: comparison of KO11 (parent) to LY01 (resistant mutant). Biotechnol Prog 19:612–623

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 71:339–349

Grandvalet C, Assad-Garcia JS, Chu-Ky S, Tollot M, Guzzo J, Gresti J, Tourdot-Marechal R (2008) Changes in membrane lipid composition in ethanol- and acid-adapted *Oenococcus oeni* cells: characterization of the *cfa* gene by heterologous complementation. Microbiology 154:2611–2619

Gustafsson C, Govindarajan S, Minshull J (2004) Codon bias and heterologous protein expression. Trends Biotechnol 22:346–353

Gutierrez T, Buszko ML, Ingram LO, Preston JF (2002) Reduction of furfural to furfuryl alcohol by ethanologenic strains of bacteria and its effect on ethanol production from xylose. Appl Biochem Biotechnol 98–100:327–340

Gutierrez T, Ingram LO, Preston JF (2006) Purification and characterization of a furfural reductase (FFR) from Escherichia coli strain LYO1–an enzyme important in the detoxification of furfural during ethanol production. J Biotechnol 121:154–164

Harvey BG, Wright ME, Quintana RL (2010) High-density renewable fuels based on the selective dimerization of pinenes. Energy Fuels 24:267–273

Heipieper HJ, de Bont JA (1994) Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl Environ Microbiol 60:4440–4444

Herrmann H, Muller C, Schmidt I, Mahnke J, Petruschka L, Hahnke K (1995) Localization and organization of phenol degradation genes of *Pseudomonas putida* strain H. Mol Gen Genet 247:240–246

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA 103:11206–11210

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807

Holms WH, Bennett PM (1971) Regulation of isocitrate dehydrogenase activity in *Escherichia coli* on adaptation to acetate. J Gen Microbiol 65:57–68

Horvath IS, Taherzadeh MJ, Niklasson C, Liden G (2001) Effects of furfural on anaerobic continuous cultivation of *Saccharomyces cerevisiae*. Biotechnol Bioeng 75:540–549

Hoschle B, Jendrossek D (2005) Utilization of geraniol is dependent on molybdenum in *Pseudomonas aeruginosa*: evidence for different metabolic routes for oxidation of geraniol and citronellol. Microbiology 151:2277–2283

Huertas MJ, Duque E, Molina L, Rossello-Mora R, Mosqueda G, Godoy P, Christensen B, Molin S, Ramos JL (2000) Tolerance to sudden organic solvent shocks by soil bacteria anal characterization of *Pseudomonas putida* strains isolated from toluene polluted sites. Environ Sci Technol 34:3395–3400

Ingram LO (1990) Ethanol tolerance in bacteria. Crit Rev Biotechnol 9:305–319

Ingram LO, Gomez PF, Lai X, Moniruzzaman M, Wood BE, Yomano LP, York SW (1998) Metabolic engineering of bacteria for ethanol production. Biotechnol Bioeng 58:204–214

Ingram LO, Vreeland NS (1980) Differential effects of ethanol and hexanol on the *Escherichia coli* cell envelope. J Bacteriol 144:481–488

Ingram LO, Vreeland NS, Eaton LC (1980) Alcohol tolerance in *Escherichia coli*. Pharmacol Biochem Behav 13(Suppl 1):191–195

Island MD, Kadner RJ (1993) Interplay between the membrane-associated UhpB and UhpC regulatory proteins. J Bacteriol 175:5028–5034

Iuchi S, Matsuda Z, Fujiwara T, Lin EC (1990) The *arcB* gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the arc modulon. Mol Microbiol 4:715–727

Jarboe LR, Grabar TB, Yomano LP, Shanmugan KT, Ingram LO (2007) Development of ethanologenic bacteria. Adv Biochem Eng Biotechnol 108:237–261

Jeffries TW, Jin YS (2000) Ethanol and thermotolerance in the bioconversion of xylose by yeasts. Adv Appl Microbiol 47:221–268

Joachimsthal EL, Rogers PL (2000) Characterization of a high-productivity recombinant strain of *Zymomonas mobilis* for ethanol production from glucose/xylose mixtures. Appl Biochem Biotechnol 84–86:343–356

Jönsson LJ, Palmqvist E, Nilvebrant N-O, Hahn-Hägerdal B (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Appl Microbiol Biotechnol 49:691–697

Junker F, Ramos JL (1999) Involvement of the cis/trans isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181:5693–5700

Kang YS, Lee Y, Jung H, Jeon CO, Madsen EL, Park W (2007) Overexpressing antioxidant enzymes enhances naphthalene biodegradation in *Pseudomonas sp*. strain As1. Microbiology 153:3246–3254

Keasling JD (2008) Synthetic biology for synthetic chemistry. ACS Chem Biol 3:64–76

Keasling JD, Chou H (2008) Metabolic engineering delivers next-generation biofuels. Nat Biotechnol 26:298–299

Keweloh H, Diefenbach R, Rehm HJ (1991) Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Arch Microbiol 157:49–53

Kieboom J, Dennis JJ, de Bont JA, Zylstra GJ (1998a) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85–91

Kieboom J, Dennis JJ, Zylstra GJ, de Bont JA (1998b) Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J Bacteriol 180:6769–6772

Kim JM, Marshall MR, Cornell JA, Preston JF, Wei CI (1995) Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. J Food Sci 60:1364

Kim K, Lee S, Lee K, Lim D (1998) Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J Bacteriol 180:3692–3696

Kirkpatrick C, Maurer LM, Oyelakin NE, Yoncheva YN, Maurer R, Slonczewski JL (2001) Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. J Bacteriol 183:6466–6477

Kishii R, Falzon L, Yoshida T, Kobayashi H, Inouye M (2007) Structural and functional studies of the HAMP domain of EnvZ, an osmosensing transmembrane histidine kinase in *Escherichia coli*. J Biol Chem 282:26401–26408

Klein-Marcuschamer D, Ajikumar PK, Stephanopoulos G (2007) Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. Trends Biotechnol 25:417–424

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Kobayashi H, Uematsu K, Hirayama H, Horikoshi K (2000) Novel toluene elimination system in a toluene-tolerant microorganism. J Bacteriol 182:6451–6455

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ (2010) Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of *Cupriavidus basilensis* HMF14. Proc Natl Acad Sci USA 107:4919–4924

Koschorreck K, Schmid RD, Urlacher VB (2009) Improving the functional expression of a *Bacillus licheniformis* laccase by random and site-directed mutagenesis. BMC Biotechnol 9:12. doi:1472-6750-9-12

Kumita H, Yamada S, Nakamura H, Shiro Y (2003) Chimeric sensory kinases containing O_2 sensor domain of FixL and histidine kinase domain from thermophile. Biochim Biophys Acta 1646:136–144

Lasko DR, Zamboni N, Sauer U (2000) Bacterial response to acetate challenge: a comparison of tolerance among species. Appl Microbiol Biotechnol 54:243–247

Lau MW, Dale BE (2009) Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST). Proc Natl Acad Sci USA 106:1368–1373

Lau PC, Wang Y, Patel A, Labbe D, Bergeron H, Brousseau R, Konishi Y, Rawlings M (1997) A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. Proc Natl Acad Sci USA 94:1453–1458

Lawford HG, Rousseau JD (1992) Effect of acetic acid on xylose conversion to ethanol by genetically engineered *E. coli*. Appl Biochem Biotechnol 34–35:185–204

Lawford HG, Rousseau JD (2003) Cellulosic fuel ethanol: alternative fermentation process designs with wild-type and recombinant *Zymomonas mobilis*. Appl Biochem Biotechnol 105–108:457–469

Lee IY, Volm TG, Rosazza JPN (1998) Decarboxylation of ferulic acid to 4-vinylguaiacol by *Bacillus pumilus* in aqueous-organic solvent two-phase systems. Enzyme Microb Technol 23:261–266

Lee J (1997) Biological conversion of lignocellulosic biomass to ethanol. J Biotechnol 56:1–24

Lee SK, Chou H, Ham TS, Lee TS, Keasling JD (2008) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr Opin Biotechnol 19:556–563

Lee YW, Jin S, Sim WS, Nester EW (1995) Genetic evidence for direct sensing of phenolic compounds by the VirA protein of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 92:12245–12249

Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2009) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101:4900–4906

Li MZ, Elledge SJ (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods 4:251–256

Lin YP, Zhang MQ, Chen BQ (2005) Research progress of ethanologenic *Zymomonas mobilis*. Wei Sheng Wu Xue Bao 45:472–477

Liu ZL, Moon J (2009) A novel NADPH-dependent aldehyde reductase gene from Saccharomyces cerevisiae NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Liu H, Sale KL, Holmes BM, Simmons BA, Singh S (2010) Understanding the interactions of cellulose with ionic liquids: a molecular dynamics study. J Phys Chem B 114:4293–4301

Liu ZL, Ma M, Song M (2009) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282:233–244

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31:345–352

Liu ZL, Moon J, Andersh AJ, Slininger PJ, Weber S (2008) Multiple gene mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and HMF by ethanologenic yeast Saccharomyces cerevisiae. Appl Microbiol Biotechnol 81:743–753

Loffeld B, Keweloh H (1996) cis/trans isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. Lipids 31:811–815

Lohrke SM, Yang H, Jin S (2001) Reconstitution of acetosyringone-mediated *Agrobacterium tumefaciens* virulence gene expression in the heterologous host *Escherichia coli*. J Bacteriol 183:3704–3711

Malpica R, Franco B, Rodriguez C, Kwon O, Georgellis D (2004) Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc Natl Acad Sci USA 101:13318–13323

Martin KV, Picataggio S, Roessler P, Verruto J, Watts K (2007) Dimethyl Octane as an advanced biofuel. Patent WO/2009/064910

Martin KV, Picataggio S, Roessler P, Verruto J, Watts K (2009) Dimethyloctane as an advanced biofuel. Patent WO/2009/064910

Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21:796–802

Mascher T, Helmann JD, Unden G (2006) Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol Mol Biol Rev 70:910–938

Michel GP, Starka J (1986) Effect of ethanol and heat stresses on the protein pattern of *Zymomonas mobilis*. J Bacteriol 165:1040–1042

Miller EN, Jarboe LR, Turner PC, Pharkya P, Yomano LP, York SW, Nunn D, Shanmugam KT, Ingram LO (2009a) Furfural inhibits growth by limiting sulfur assimilation in ethanologenic *Escherichia coli* strain LY180. Appl Environ Microbiol 75:6132–6141

Miller EN, Jarboe LR, Yomano LP, York SW, Shanmugam KT, Ingram LO (2009b) Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. Appl Environ Microbiol 75:4315–4323

Mitchell RJ, Yun-Jie Lee, Sun-Mi Lee, Joo-Myung Ahn, Dae Haeng Cho, Youngsoon Um, Yong Hwan Kim, Man Bock Gu, Sang. B-I (2008) Toxic response of *Clostridium beijerinckii* and *Escherichia coli* to lignin hydrolysate-related compounds. Paper presented at the AICHE. http://aiche.confex.com/aiche/s08/techprogram/P108620.HTM. Accessed on 30 July 2011

Moken MC, McMurry LM, Levy SB (1997) Selection of multiple-antibiotic-resistant (*mar*) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. Antimicrob Agents Chemother 41:2770–2772

Mukhopadhyay A, Redding AM, Rutherford BJ, Keasling JD (2008) Importance of systems biology in engineering microbes for biofuel production. Curr Opin Biotechnol 19:228–234

Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD (2006) High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95:684–691

Ng LC, Shingler V, Sze CC, Poh CL (1994) Cloning and sequences of the first eight genes of the chromosomally encoded (methyl) phenol degradation pathway from *Pseudomonas putida* P35X. Gene 151:29–36

Okusu H, Ma D, Nikaido H (1996) AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol 178:306–308

Osman YA, Ingram LO (1985) Mechanism of ethanol inhibition of fermentation in *Zymomonas mobilis* CP4. J Bacteriol 164:173–180

Palmqvist E, Hahn-Hagerdal B (2000a) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol 74:17–24

Palmqvist E, Hahn-Hagerdal B (2000b) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25–33

Papoutsakis ET (2008) Engineering solventogenic clostridia. Curr Opin Biotechnol 19:420–429

Park S, Kazlauskas RJ (2003) Biocatalysis in ionic liquids - advantages beyond green technology. Curr Opin Biotechnol 14:432–437

Peralta-Yahya PP, Keasling JD (2010) Advanced biofuel production in microbes. Biotechnol J 5:147–162

Perez JM, Arenas FA, Pradenas GA, Sandoval JM, Vasquez CC (2008) *Escherichia* coli YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. J Biol Chem 283:7346–7353

Petersson A, Almeida JR, Modig T, Karhumaa K, Hahn-Hagerdal B, Gorwa-Grauslund MF, Liden G (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene conveys HMF tolerance. Yeast 23:455–464

Pfleger BF, Pitera DJ, Smolke CD, Keasling JD (2006) Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat Biotechnol 24:1027–1032

Phoenix P, Keane A, Patel A, Bergeron H, Ghoshal S, Lau PC (2003) Characterization of a new solvent-responsive gene locus in *Pseudomonas putida* F1 and its functionalization as a versatile biosensor. Environ Microbiol 5:1309–1327

Pienkos PT, Zhang M (2009) Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. Cellulose 16:743–762

Pitera DJ, Paddon CJ, Newman JD, Keasling JD (2007) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metab Eng 9:193–207

Polen T, Rittmann D, Wendisch VF, Sahm H (2003) DNA Microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. Appl Environ Microbiol 69:1759–1774

Quan J, Tian J (2009) Circular polymerase extension cloning of complex gene libraries and pathways. PLoS ONE 4(7):e6441. doi:10.1371/journal.pone.0006441

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006) The path forward for biofuels and biomaterials. Science 311:484–489

Ramos JL, Duque E, Gallegos M-T, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56:743–768

Ramos JL, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323–3329

Ranatunga TD, Jervis J, Helm RF, McMillan JD, Hatzis C (1997) Identification of inhibitory components toxic toward *Zymomonas mobilis* CP4(pZB5) xylose fermentation. Appl Biochem Biotechnol 67:185–198

Rogers PL, Goodman AE, Heyes RH (1984) Zymomonas ethanol fermentations. Microbiol Sci 1:133–136

Roling WF, Milner MG, Jones DM, Lee K, Daniel F, Swannell RJ, Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl Environ Microbiol 68:5537–5548

Ruhl J, Schmid A, Blank LM (2009) Selected *Pseudomonas putida* strains able to grow in the presence of high butanol concentrations. Appl Environ Microbiol 75:4653–4656

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD (2010) Functional genomic study of exogenous n-butanol stress in *Escherichia coli*. Appl Environ Microbiol 76:1935–1945

Ryder JA (2009) Jet fuel compositions. Patent 12/393,024

Sakamoto T, Murata N (2002) Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. Curr Opin Microbiol 5:208–210

Salis H, Tamsir A, Voigt C (2009) Engineering bacterial signals and sensors. Contrib Microbiol 16:194–225

Sariaslani FS (2007) Development of a combined biological and chemical process for production of industrial aromatics from renewable resources. Annu Rev Microbiol 61:51–69

Sauer M, Marx H, Mattanovich D (2008) Microbial production of 1,3-propanediol. Recent Pat Biotechnol 2:191–197

Saxena RK, Anand P, Saran S, Isar J (2009) Microbial production of 1,3-propanediol: recent developments and emerging opportunities. Biotechnol Adv 27:895–913

Shetty RP, Endy D, Knight TF Jr (2008) Engineering BioBrick vectors from BioBrick parts. J Biol Eng 2:5. doi:1754-1611-2-5

Sikkema J, Debont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201–222

Silveira MG, Baumgartner M, Rombouts FM, Abee T (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl Environ Microbiol 70:2748–2755

Simmons BA, Loque D, Blanch HW (2008) Next-generation biomass feedstocks for biofuel production. Genome Biol 9:242

Singh S, Simmons BA, Vogel KP (2009) Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. Biotechnol Bioeng 104:68–75

Smolke CD (2009) Gene expression tools for metabolic pathway engineering. In: Smolke CD (ed) The metabolic pathway engineering handbook: tools and applications. CRC Press, San Diego

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H (2004) Toward a systems approach to understanding plant cell walls. Science 306:2206–2211

Sommer MO, Church GM, Dantas G (2010) A functional metagenomic approach for expanding the synthetic biology toolbox for biomass conversion. Mol Syst Biol 6:360. doi:msb201016

Sperandio V, Torres AG, Kaper JB (2002) Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol Microbiol 43:809–821

Spormann AM, Widdel F (2000) Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11:85–105

Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463:559–562

Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69:183–215

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11

Swatloski RP, Spear SK, Holbrey JD, Rogers RD (2002) Dissolution of cellulose with ionic liquids. J Am Chem Soc 124:4974–4975

Tittabutr P, Payakapong W, Teaumroong N, Boonkerd N, Singleton PW, Borthakur D (2006) A histidine kinase sensor protein gene is necessary for induction of low pH tolerance in *Sinorhizobium sp.* strain BL3. Antonie Leeuwenhoek 89:125–134

Tomas CA, Beamish J, Papoutsakis ET (2004) Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol 186:2006–2018

Tomas CA, Welker NE, Papoutsakis ET (2003) Overexpression *of groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 69:4951–4965

Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, Saija A, Mazzanti G, Bisignano G (2005) Mechanisms of antibacterial action of three monoterpenes. Antimicrob Agents Chemother 49:2474–2478

Tyo KE, Ajikumar PK, Stephanopoulos G (2009) Stabilized gene duplication enables long-term selection-free heterologous pathway expression. Nat Biotechnol 27:760–765

Underwood SA, Buszko ML, Shanmugam KT, Ingram LO (2004) Lack of protective osmolytes limits final cell density and volumetric productivity of ethanologenic *Escherichia coli* KO11 during xylose fermentation. Appl Environ Microbiol 70:2734–2740

Utsumi R, Brissette RE, Rampersaud A, Forst SA, Oosawa K, Inouye M (1989) Activation of bacterial porin gene expression by a chimeric signal transducer in response to aspartate. Science 245:1246–1249

van Beilen JB, Panke S, Lucchini S, Franchini AG, Rothlisberger M, Witholt B (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. Microbiology 147:1621–1630

Van Dyk TK (2008) Bacterial efflux transport in biotechnology. Adv Appl Microbiol 63:231–247
Van Dyk TK, Templeton LJ, Cantera KA, Sharpe PL, Sariaslani FS (2004) Characterization of the *Escherichia coli* AaeAB efflux pump: a metabolic relief valve? J Bacteriol 186:7196–7204
Vemuri GN, Altman E, Sangurdekar DP, Khodursky AB, Eiteman MA (2006) Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. Appl Environ Microbiol 72:3653–3661
Verhoef S, Wierckx N, Westerhof RG, de Winde JH, Ruijssenaars HJ (2009) Bioproduction of p-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. Appl Environ Microbiol 75:931–936
Wackett LP (2008) Biomass to fuels via microbial transformations. Curr Opin Chem Biol 12:187–193
Wagner S, Baars L, Ytterberg AJ, Klussmeier A, Wagner CS, Nord O, Nygren PA, van Wijk KJ, de Gier JW (2007) Consequences of membrane protein overexpression in *Escherichia coli*. Mol Cell Proteomics 6:1527–1550
Wagner S, Klepsch MM, Schlegel S, Appel A, Draheim R, Tarry M, Hogbom M, van Wijk KJ, Slotboom DJ, Persson JO, de Gier JW (2008) Tuning *Escherichia coli* for membrane protein overexpression. Proc Natl Acad Sci USA 105:14371–14376
Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63
Warnecke T, Gill RT (2005) Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. Microb Cell Fact 4:25. doi:1475-2859-4-25
Welch M, Govindarajan S, Ness JE, Villalobos A, Gurney A, Minshull J, Gustafsson C (2009) Design parameters to control synthetic gene expression in *Escherichia coli*. PLoS ONE 4(9): e7002. doi:10.1371/journal.pone.0007002
Wierckx N, Koopman F, Bandounas L, de Winde JH, Ruijssenaars HJ (2010) Isolation and characterization of *Cupriavidus basilensis* HMF14 for biological removal of inhibitors from lignocellulosic hydrolysate. Microb Biotechnol 3:336–343
Wright JS III, Kadner RJ (2001) The phosphoryl transfer domain of UhpB interacts with the response regulator UhpA. J Bacteriol 183:3149–3159
Wright JS, Olekhnovich IN, Touchie G, Kadner RJ (2000) The histidine kinase domain of UhpB inhibits UhpA action at the *Escherichia coli uhpT* promoter. J Bacteriol 182:6279–6286
Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY, Mitchinson C, Saddler JN (2009) Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies. Biotechnol Prog 25:333–339
Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 20:132–138
Zaldivar J, Ingram LO (1999) Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01. Biotechnol Bioeng 66:203–210
Zaldivar J, Martinez A, Ingram LO (1999) Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol Bioeng 65:24–33
Zaldivar J, Martinez A, Ingram LO (2000) Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol Bioeng 68:524–530
Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34
Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods 3:623–628
Zeng A, Biebl H (2010) Bulk chemicals from biotechnology: the case of 1,3-propanediol production and the new trends. Adv Biochem Eng Biotechnol 74:239–258
Zhang Y, Zhu Y, Li Y (2009) The importance of engineering physiological functionality into microbes. Trends Biotechnol 27:664–672

Metabolomics for Ethanologenic Yeast

Ying-Jin Yuan, Ming-Zhu Ding, Jin-Mei Xia, and Jing-Sheng Cheng

Contents

1 Introduction 240
2 GC-MS Based Research Strategy 242
2.1 Principles of GC-MS Analysis 242
2.2 Basic Sample Preparation 244
2.3 Identification and Quantification of Metabolites 244
2.4 Metabolomic Analysis of Yeast with Different Inoculum Densities 245
2.5 Metabolomic Study of Yeast During Industrial Continuous and Batch Fermentation 246
2.6 Metabolomic Analysis of Yeast Under Vacuum Fermentation 248
3 LC-MS Based Research Strategy 249
3.1 Fundamentals of LC-MS Analysis 249
3.2 Lipidomics 250
3.3 Lipidomic Study of Yeast in Response to Inhibitors 250
4 NMR 252
5 Data Mining 252
5.1 Unsupervised 252
5.2 Supervised 253
6 Conclusion and Perspectives 253
References 254

Abstract Metabolomics-based studies have been applied widely to improve our understanding of molecular mechanisms of yeast stress response as well as to seek foundational basis for further optimization of fermentation processes. In this chapter, the basic principles of metabolomic approaches including sample preparation, metabolomic analysis, metabolite identification and quantification, data

Y.-J. Yuan (✉) • M.-Z. Ding • J.-M. Xia • J.-S. Cheng
Key Laboratory of Systems Bioengineering, Ministry of Education; Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
e-mail: yjyuan@tju.edu.cn

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_10,

mining, and biological interpretation are summarized, emphasizing on the gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS) based strategies. The major applications of metabolomics on ethanologenic yeast during ethanol production are highlighted, such as stress response to high cell density, inhibitory compounds in the lignocellulosic hydrolysates, different (batch and continuous) fermentation modes, and vacuum fermentation conditions.

1 Introduction

Ethanol production from various lignocellulosic materials has received increasing attention recently due to the concerns on limited fossil energy and environmental consequences of using fossil fuels (Hill et al. 2006). During the process of ethanol production from lignocellulosic feedstock, some key factors, such as the inoculation density, the inhibitors in the hydrolysates and the separation of final products by vacuum condition, can cause various stresses to cells. Inhibitory compounds, such as furan derivatives, phenolic compounds, and weak acids generated during pre-treatment of lignocelluloses, cause stresses on yeast cells (Palmqvist and Hahn-Hagerdal 2000; Klinke et al. 2004; Liu and Blaschek 2010). High cell-density fermentation in industry is a promising strategy to improve productivity, save fermentation time, and reduce volume of fermenter. However, fermentation with high cell density could also give rise to the depletion of essential nutrients and accumulation of inhibitory products, which consequently influence the fermentation process and the quality of final products (Van Hoek et al. 2000). In addition, stress conditions from final product ethanol also influence the economics of biofuel production process by *Saccharomyces cerevisiae* (Attfield 1997). Direct separation of ethanol from the fermentation broth using vacuum fermentation strategy has been used to decrease the inhibitory effects generated from ethanol and other volatile compounds (Cysewski and Wilke 1977). However, other stresses such as negative pressure, oxygen deficiency, and accumulation of nonvolatile toxic metabolites still exist during vacuum fermentation. These stress effects are often synergistic, affecting yeast cells more severely than any single one when present together in the broth, leading to reduced yeast viability and vigor as well as lower ethanol yield (Bai et al. 2008). However, currently the molecular-level understanding of stress effects is still insufficient.

Genomics, transcriptomics, and proteomics have been widely applied to study the yeast responses to stressful fermentation conditions (Gasch et al. 2000; Cheng et al. 2009a; Lin et al. 2009; Li et al. 2010a, 2010b; Li and Yuan 2010c). Since metabolites represent the distal readout of cellular state as well as associated physiology, the changes at metabolome level are expected to be amplified relative to changes in transcriptome and proteome. Metabolomics, which is a systematic approach in describing the physiological state through quantitatively analyzing the metabolome of living cells, is expected to play a complementary role to other

'omics' (Urbanczyk-Wochniak et al. 2003). In addition, multiple metabolic pathways of cells are often affected by the same stress condition simultaneously. Therefore, only when the metabolome is characterized as a whole, the pathways perturbed by the stress could be identified with a high degree of certainty (Garcia et al. 2008). Thus, it is more suitable to characterize the cellular stress responses by metabolomic analysis. Over the last decade, the number of publications in the field of metabolomics rises rapidly. Since the first 'metabolome' article published in 1998 by Tweeddale et al. (1998), the number of papers in this field increased significantly (Fig. 1). Moreover, the journal *Metabolomics* (Springer) was launched in 2005, and received its first and second impact factor of 3.254 and 3.871 from Thomson Reuters, respectively. This journal publishes papers involved in development and application of metabolomics, and comparative integrated studies with other 'omics'. Some of recent studies have demonstrated that metabolomics is a valuable emerging tool to study phenotypes and their changes caused by environmental influences or changes in genotype.

In recent years, our laboratory has applied metabolomics to study the behaviors of ethanologenic yeast in response to various stress conditions, and some interesting results have been obtained (Xia and Yuan 2009; Ding et al. 2009a, 2009b, 2010a, 2010b, 2011; Zhou et al. 2010; Xia et al. 2010). Metabolomic studies improve our understanding of molecular mechanisms of yeast responses to stresses, and help us to seek foundational basis for further optimizing the fermentation process. They were summarized in this chapter along with some other excellent applications of metabolomics in yeast stress response by other groups. Altogether, these studies can be classified into five major categories based on the analytical instruments: (1) gas chromatography coupled with mass spectrometry (GC-MS) based metabolomic analyses that have been used to investigate the metabolic responses of yeast strains

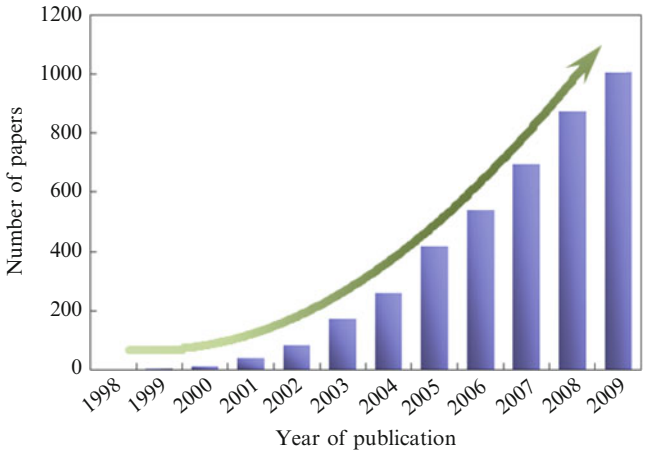

Fig. 1 Bibliometric analysis of the recent metabolomics literature. The growth in total publications related to metabolomics by topics searching using 'metabolom' or 'metabonom' from Web of Science

to stress conditions such as very high gravity fermentation and high inoculum density (Devantier et al. 2005; Ding et al. 2009a); (2) metabolomic analysis using liquid chromatography coupled to mass spectrometry (LC-MS) that has revealed the impact of heat stress (Cowart et al. 2010), cadmium stress (Lafaye et al. 2005), acetic acid, phenol, and furfural stress (Xia and Yuan 2009) on lipid species; (3) metabolome analyses by capillary electrophoresis mass spectrometry (CE-MS) that have revealed the intracellular responses caused by polyethylene glycol (Kawai et al. 2009) and cadmium exposure (Tanaka et al. 2007); (4) nuclear magnetic resonance (NMR) analysis that has identified a number of inter-linked metabolic pathways that exhibit oxidative stress-dependent regulatory patterns of (Weeks et al. 2006); and (5) fourier transform infrared Spectroscopy (FTIR) technology that has been used to study stress responses induced by four chemical compounds (ethanol, sodium hypochlorite, sodium chloride and sulfur dioxide) at different concentrations (Corte et al. 2010). Metabolomics has become more extensively applied in studying yeast stress responses.

The common strategies of major metabolomic approaches typically include the following steps: sample preparation (e.g., sampling, quenching, and extraction), metabolomic analysis [e.g., by gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS)], metabolite identification, quantification and biological interpretation (Fig. 2). In this chapter, we will emphasize the principles and recent applications of these strategies towards metabolomic analysis for ethanologenic yeast.

2 GC-MS Based Research Strategy

Gas chromatography coupled with mass spectrometry (GC-MS) has become one of the most popular analytical platforms for metabolomic analysis. The essentials for GC-MS analysis and its extensive applications on yeast cells during the process of ethanol production from lignocellulosic materials are highlighted here.

2.1 Principles of GC-MS Analysis

GC-MS is one of the most popular analytical platforms for metabolomic analysis, which is generally performed using quadrupole (Q-MS) or time-of-flight (TOF-MS). The major advantage of GC-MS for metabolomics is the availability of both commercially and publicly available spectral libraries (Halket et al. 2005). GC-MS is applicable for profiling several hundred compounds of diverse chemical classes, including volatile metabolites (such as alcohols, monoterpenes, and esters), as well as non-volatile polar metabolites (e.g. amino acids, sugars, and lipids) through derivatization by methylation or trimethylsilylation. The GC-MS strategy is limited in the molecular mass that it can measure, and thermolabile ones are necessarily missed. Quadrupole mass analyzers present some advantages in metabolite

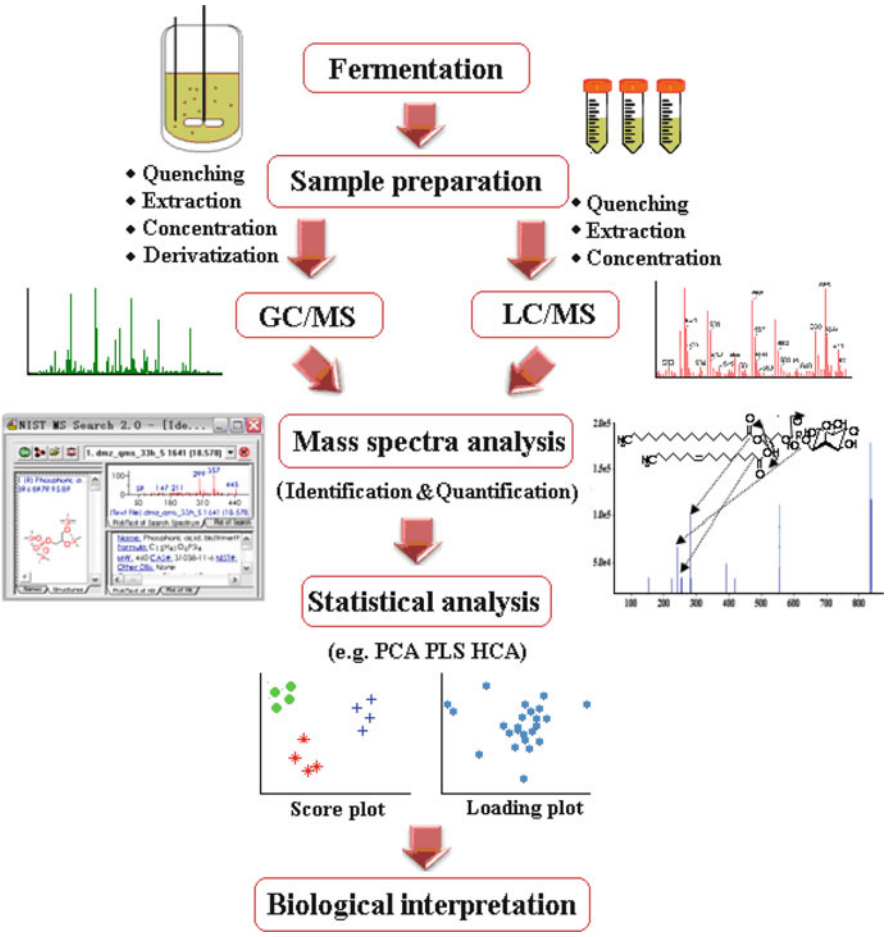

Fig. 2 A schematic illustration of methods and procedures of metabolomics

analysis, such as higher sensitivity, repeatability, and large dynamic range for quantitative analysis (Nielsen et al. 2003). More recently, the combination of GC with TOF/MS offers an attractive supplement to quadrupole instruments and has provided greater mass accuracy. The GC-TOF/MS provides high scan speed that is compatible with ultrafast GC-MS, and has the potential ability to profile complex mixtures in less time, thereby increasing laboratory throughput. GC × GC-MS is another increasing popular strategy through which metabolites in samples can be better separated in a single analytic run (Van Mispelaar et al. 2003). The method involving flow injection analysis using direct infusion into electrospray ionization (ESI) coupled with TOF or fourier transform ion cyclotron resonance (FT-ICR) MS analysis is also becoming popular (Ivanova et al. 2001; Allen et al. 2003).

2.2 Basic Sample Preparation

To arrest the enzymatic activity and minimize the degradation of metabolites, cells must be quenched instantaneously and completely when they are sampled for metabolomic analysis. Dramatic changes in temperature or pH, such as rapid sampling into liquid nitrogen (Hans et al. 2001), or in a cold-buffered methanol solution, are commonly used quenching methods (Gonzalez et al. 1997). During the extraction of intracellular metabolites following the quenching step, it is important that the extraction conditions must be carefully chosen to avoid any chemical modification or degradation, and to achieve minimal loss of metabolites. Commonly used methods of intracellular metabolite extraction for microorganisms include boiling ethanol (Gonzalez et al. 1997; Hajjaj et al. 1998), cold methanol (50–100%) (Maharjan and Ferenci 2003; Wittmann et al. 2004), and chloroform/methanol/water mixture (de Koning and van Dam 1992). As most of these extraction methods will unavoidably result in a high degree of sample dilution, the concentration of a final extracted sample is often low. Lyophilization with the advantage of deep-freezing and dehydration is commonly used to concentrate the extracted samples for final analysis. Most of the metabolites are stabilized with this non-aggressive technology as they are dried from the frozen solution. Finally, it has been shown that metabolites are more stable when stored free of water. When kept in a cold, dry, and neutral atmosphere, and for some metabolites in the dark, the samples can be stored for a very long time without loss of metabolites (Villas-Bôas et al. 2005).

For metabolomic analysis by GC-MS, modification of polar functional groups (i.e., derivatization) is necessary to facilitate various classes of compounds volatile and thermally stable. The derivatization procedure of methoximation followed by trimethylsilylation is often applied to analyze metabolome with GC-MS (Roessner et al. 2000)**.** The most commonly used reagent for methoxymation is methoxylamine hydrochloride, dissolved in pyridine solution. In silylation, the active hydrogen in functional groups (e.g., hydroxylic and carboxylic groups or amines) can be replaced by an alkylsilyl group, primarily trimethylsilyl (TMS). Silyl derivatives show a better thermal stability and higher volatility, thus can produce more distinct mass spectra. Comparable in silyl donor strength among the predominantly used reagents for trimethylsilylation, *N*-methyl-*N*-(trimetylsilyl)trifluoroacetamide (MSTFA) gives the best result with the broadest range of chemical compounds, produces the least by-products, and is therefore suitable for GC-MS analysis (Roessner et al. 2000).

2.3 Identification and Quantification of Metabolites

Metabolites can be identified by comparing fragment patterns and retention index with those of standard compounds in databases, such as the National Institute of

Standards and Technology (NIST) library, the Golm Metabolome Database (GMD. http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) (Kopka et al. 2005), and the METLIN metabolomic database (http://metlin.scripps.edu/) (Smith et al. 2005).

Quantification of compounds is carried out by comparing peak area of each individual metabolite with that of internal standard. The interested metabolites are integrated with the related metabolic pathways for further interpretation. Development of an array of publicly available bioinformatic tools in recent years has dramatically improved the integration of metabolomics and other 'omics' data. The widely used pathway databases and pathway viewers, for example, Kyoto Encyclopedia of Genes and Genomes (KEGG. http://www.genome.ad.jp/kegg/), *Saccharomyces* genome database (http://pathway.yeastgenome.org/), BioCyc (http://biocyc.org) (Paley and Karp 2006), and MetaCyc (http://metacyc.org/) (Caspi et al. 2006), are useful resources in investigating cell responses to stress conditions.

2.4 *Metabolomic Analysis of Yeast with Different Inoculum Densities*

During the industrial fermentation, inoculum density is one of the most important factors that influence the fermentation process, thus the quality of final products (Sen and Swaminathan 2004). High cell-density fermentation can improve the productivity of final products and shorten fermentation time. However, yeast cells may encounter different kinds of stresses when grew in high cell-density fermentation conditions. One of the stresses is that most inhibitory compounds would be induced by high cell population and they could affect yeast metabolism either separately or cooperatively. In addition, the quick depletion of essential nutrients in high cell-density culture medium may also limit the production of ethanol. Ding et al. (2009a) recently employed the metabolomics to characterize the stress responses and regulations depending on inoculum density during ethanol fermentation.

Metabolomic analysis was carried out on industrial yeast during fermentations with five different inoculum densities (1 g/l, 5 g/l, 10 g/l, 20 g/l and 40 g/l). Quenching was performed by 60% methanol (−40 °C, v/v). The 50% methanol aqueous solution was used for metabolite extraction by freeze thawing in liquid nitrogen. Succinic d_4 acid was added as the internal standard before lyophilization. The derivatization procedure including methoximation by methoxamine hydrochloride and trimethylsilylation by MSTFA both at 40°C for 80 min was used. GC-TOF/MS (Waters Corp., USA) was employed for detection. The mass chromatography was analyzed by Masslynx software (Version 4.1, Waters Corp., USA) and the identification was employed by comparing the mass fragments to the NIST library and Golm Metabolome Database. All the peak areas were normalized against that of the internal standard for further analysis.

Metabolomic analysis revealed that inoculum size had profound influences on yeast metabolism in ethanol fermentation. Some key metabolic pathways were affected, including central carbon metabolism (glycerol, phosphoric acid, and succinate), amino acid metabolism (glycine, isoleucine, and proline), as well as membrane structure and function related metabolites (palmitoleic acid, myo-inositol and ethanolamine). Most of the detected metabolites, especially succinate, glycerol, isoleucine, and proline, represented an abrupt shift from fermentations with 20 g/l inoculum to 40 g/l inoculum, indicating that inoculum density of higher than 20 g/l imposed significantly stresses on yeast metabolism. These results showed that the change of the inoculum size could cause significant changes of the concentration of key intermediates in glycolysis and TCA cycle. The elevated levels of pyruvate and declined levels of TCA intermediates were found as the inoculum size increased, suggesting that the fermentation activity increased in higher cell-density fermentation. Moreover, high inoculum size was accompanied by rapid accumulation of most amino acids, which seemed to be responsible for the reduction of their precursors involving in glycolysis and TCA cycle. In addition, the membrane structure of yeast was also affected by inoculum density. The lower levels of myo-inositol and ethanolamine under higher inoculum size might be related to the synthesis of phosphatidylinositol (PI) and phosphatidylethanolamine (PE), respectively, which affected the stability of bilayer and fluidity of cell membrane. High inoculum size also triggered an induction of palmitoleic acid to protect cells from damage and keep better viability to survive under the condition that contained accumulated toxic products.

Furthermore, the results showed that high cell-density fermentation imposed a substantially stressful environment for yeast growth and metabolism. Glycerol and proline, for their significant accumulation in fermentation with an inoculum size of 40 g/l, were considered as important protectors for yeast to survive under the high cell-density condition. Induction of the intracellular accumulation of glycerol and proline has been found to protect yeast cells from damages which were caused by various stress conditions, such as osmotic, thermal, oxidative, freezing, and ethanol stress (Brewster et al. 1993; Hohmann 2002; Takagi 2008). In addition, sharp accumulation of intracellular glycerol was reported to maintain the redox balance by reoxidizing the surplus of NADH and $FADH_2$ (Van Dijken and Scheffers 1986). Therefore, it was speculative that the redox balance may be destroyed by the limited availability of oxygen during high cell-density fermentation. These findings were of great importance for optimizing the inoculation density in industrial ethanol fermentation.

2.5 Metabolomic Study of Yeast During Industrial Continuous and Batch Fermentation

In industrial ethanol fermentation, metabolism of yeast cells was significantly influenced by environmental stress, such as high sugar concentration, high

temperature, oxygen stress, osmotic stress, nutrient deficiency, and contamination, as well as ethanol accumulation. Many biochemical events, including cell stress sensing, stress protectants accumulation, signal transduction, transcription, and translation, were involved in the yeast responses to stress conditions (Devantier et al. 2005). Comparative metabolomic analysis was also used to compare industrial continuous and batch fermentation processes and to seek insights into the differentially metabolic characteristics of *S. cerevisiae* in response to different fermentation conditions (Ding et al. 2009b). During the two industrial processes, the environmental conditions and the physiological responses of cells are different. Thus, it is necessary to understand the metabolic regulation in yeast cells in order to manipulate the industrial fermentation process efficiently and to improve the ethanol production.

Both the continuous and batch fermentations were carried out using industrial strain *S. cerevisiae*. The fermentation processes lasted about 60 h, and seven samples were collected from the two processes for metabolomic analysis. The metabolites were extracted by methanol/chloroform/water. Two-stage chemical derivatization was performed on the extracted metabolites prior to analysis by GC-TOF/MS. First, the sample was oximated with methoxamine hydrochloride, incubating at 30°C for 90 min. Then, it was trimethylsilylated with MSTFA at 37°C for 30 min. The oven temperature of GC was programmed as: 70°C for 2 min, then increased to 290°C at a rate of 8°C/min and holding for 3 min. Multivariate statistical analysis was carried out by principal component analysis (PCA) using Markerlynx software (Waters Corp., USA).

A strategy of GC-TOF/MS-based metabolomics combined with multivariate statistical analysis was used in this study to provide metabolic profiles of industrial *S. cerevisiae* during the continuous and batch fermentation. The samples from continuous and batch fermentation were clearly separated by PCA in the score plot, indicating that the industrial *S. cerevisiae* displayed distinctly metabolic characteristics under different fermentation environments. It was also found that glycerol and phosphoric acid were the most important metabolites that distinguishes the continuous from the batch fermentations on the loadings plot.

The industrial ethanol fermentation process in general contains three phases, including seed phase, main phase and final phase according to sugar consumption and ethanol production. In this study, continuous and batch fermentations were both clearly distinguished into these three phases by PCA. Metabolites associated with glycolysis pathway (phosphoric acid, lactic acid and glycerol), intermediates of TCA cycle (citrate and malate), and amino acids (glycine and glutamine) contributed to the cluster formation significantly. Glycerol and phosphoric acid were principally responsible for discriminating seed, main, and final phases of continuous fermentation, while lactic acid and glycerol contributed mostly to inform different phases of batch fermentation. The results from this study could serve as a starting point for further investigation into the metabolism network of *S. cerevisiae* under complex industrial fermentation conditions.

2.6 Metabolomic Analysis of Yeast Under Vacuum Fermentation

In ethanol fermentation, accumulation of ethanol influences cell growth and viability, glucose and amino acid transportation, as well as membrane structure and function (Ingram and Buttke 1984; Attfield 1997). Vacuum fermentation that continuously removes volatile inhibitory products from fermentation medium can therefore eliminate the end product inhibition effectively (Maiorella et al. 1983). During vacuum ethanol fermentation, yeast cells are also subjected to a variety of environmental stresses that have not been well documented. These stresses ranged from physical (e.g., intracellular and extracellular pressure difference) to chemical stress (e.g., oxygen deficiency and nonvolatile toxic metabolites accumulation) (Cheng et al. 2009b). Metabolomic study on *S. cerevisiae* was performed as the cells underwent a vacuum adaptive evolution recently (Ding et al. 2010a). The regulatory mechanisms of metabolic pathways in adaptation to the extreme vacuum condition have been illustrated.

The first round of repeated vacuum fermentations (i.e., VFI) was conducted under pressure of 50 $\pm$ 5 mbar continuously for 30 cycles. One cycle was defined as when glucose content decreased from 100 g/l to about 20 g/l. When the glucose content dropped to about 20 g/l in the broth, YPD medium was added to keep the glucose concentration and working volume as 100 g/l and 5 l, respectively. The second round of repeated vacuum fermentations (i.e., VFII) were performed under the same condition of the VFI used the vacuum-adapted yeast strain from the VFI.

Metabolomic analysis revealed that metabolic states of cells changed significantly during the adaptive evolution process. Metabolites related to stress response, including glycerol, trehalose, myo-inositol and glutamate, might be involved in response to the vacuum stress, while their decreased levels afterward indicated that the yeast cells adapted to vacuum condition as the fermentation progressed. Furthermore, glycolysis and TCA cycle intermediates were enhanced, whereas glycerol biosynthesis was depressed by vacuum. The decreases of most amino acids might be related to increases of glycolysis and TCA cycle intermediates as the VFI progressed.

In addition, the vacuum-adapted yeast that re-cultured under the vacuum condition displayed distinct metabolic characteristics. The lower levels of glycerol, myo-inositol, trehalose, and glutamate in the VFII than in the VFI indicated that the adapted yeast presented better tolerance to the vacuum stress condition. The results also indicated that yeast cells exposed previously to the vacuum stress adjusted their metabolism rapidly by initiating stress responses fast, including rapid synthesis of protective molecules (e.g., glycerol and trehalose) and activation of signal transduction pathways, which helped cells to survive and recover to normal cellular activities (Ding et al. 2010).

3 LC-MS Based Research Strategy

Liquid chromatography coupled to mass spectrometry (LC-MS) strategy has been widely used in metabolomic study. The development of LC-MS has facilitated the development of lipidomics (Gaspar et al. 2007). LC-MS based lipidomic studies have potential to provide insight into physiological responses of ethanologenic yeast during ethanol production.

3.1 Fundamentals of LC-MS Analysis

Different from GC-MS, LC-MS analysis does not require the molecules to be volatile and is thus receiving more attentions recently. The sample preparation in LC-MS strategy is similar with GC-MS except that derivatization is not necessary. The fractionation of metabolites in GC is realized based on their different volatilities, while the separation of molecules in LC is due to their different polarities.

When the fractionation is realized by LC, the ionization mode frequently used is ESI and atmospheric pressure chemical ionization (APCI). In fact, one major advantage of electrospray ionization (ESI) is that it facilitates the coupling of liquid chromatography to mass spectrometers. The low sample consumption, the good sensitivity, and the ease to operate for ESI make it much more popular than other ionization modes, such as thermospray, fast-atom bombardment (FAB), particle-beam, and APCI (Niessen 1998). ESI has positive and negative ionization modes. Metabolites are usually detected under the ESI- after losing an H^+ or under the ESI + after gaining H^+, NH_4^+, Na^+, or K^+. Because the formation of potential adduct is a problem for all four ions under the ESI+, therefore, the ESI− is preferred in many occasions.

The properties of columns influence the resolution of LC-MS instruments whereas both column and the mass spectrometer influence sensitivity of the system. Ion trap mass spectrometers can realize several tandem MS analyses in series using one ion trap and are widely used to fragment selected ions to aid the structural analyzing process. The assignment of mass signals detected via LC-MS approach relies on the combination of three parameters: accurate mass, retention time, and MS/MS fragmentation data. Quantification of compounds can be realized by using standards and comparing the peak area of a certain metabolite with a standard.

Metabolites with a higher polarity are usually eluted at solvent front on LC columns, and thus are analyzed with low resolution. For the analysis of polar metabolites, reverse-phase columns together with hydrophilic interaction chromatography (HILIC) columns have been developed. High-pressure columns are also developed to be used in ultrahigh-pressure liquid chromatography (UPLC)-MS system (Plumb et al. 2006), which offers improved chromatographic fractionation of samples and thus reduce the ionization repression effect of the ESI.

3.2 Lipidomics

Lipidomic approach can be used easily to quantitatively characterize all lipids. As a branch of metabolomics, lipidomic strategy focuses on system level analysis of lipids and factors that intact with lipids. The development of yeast lipidomics was facilitated by the development of high-throughput equipment, especially LC-MS (Gaspar et al. 2007). Sampling, quenching, extraction, concentration and LC-tandem MS strategies (such as di-quadrupole MS/MS, and triple quadrupole MS/MS) are typical procedures for lipidomics research (Han and Yuan 2009). For phospholipid characterization, an ion trap mass spectrometer is typically used to perform data dependent MS^n scanning (Yang et al. 2007). In data-dependent MS^n acquisition, when one ion is detected in MS, the mass spectrometer automatically fragments this ion and acquires its product ion mass spectrum (MS^2). Similarly, a second order product ion (MS^3) mass spectrum can be generated for the base peak ion in the MS^2 spectrum. Both the acyl chains and the head groups of phospholipid molecules can form characteristic fragments and the structures of phospholipids can be interpreted based on the fragmentation information.

Membrane lipids are the most adaptable molecules in response to environmental changes and were the targets in stress adaptation (Russell et al. 1995). The composition changes of both the acyl chains and the polar head groups can alter the packing arrangements of the lipids, and thus can affect the bilayer stability and fluidity, even the lipid-protein interactions. Research by Mannazzu et al. (2008) and Lei et al. (2007) revealed that lipid composition and membrane integrity played important roles in the adaptation to unfavorable conditions in yeast. The variety of polar groups and the length or the unsaturation level for the associated fatty acids of lipids made the bilayer matrix of cell membranes very complex (Wolf and Quinn 2008), which demands the profiling of the entire spectrum of lipids or lipidome.

3.3 Lipidomic Study of Yeast in Response to Inhibitors

When lingocellulose is hydrolyzed into fermentable sugars by physical, chemical, and biological pre-treatments, various inhibitory compounds are inevitably produced (Mosier et al. 2005). Furan aldehydes and ketones from sugar degradation, aromatics from lignin degradation, and low molecular weight organic acids from hemicellulose hydrolysis were typical inhibitors derived from lignocellulose hydrolysate (Palmqvist and Hahn-Hagerdal 2000; Martinez et al. 2001; Klinke et al. 2004; Liu and Blaschek 2010).

The changes of membrane lipids of four *S. cerevisiae* strains, including an industrial strain (SC), a furfural-tolerant strain (SCF), a phenol-tolerant strain (SCP), and an acetic acid-tolerant strain (SCA), were investigated using LC-ESI/MS^n technique under the stress of furfural, phenol, and acetic acid (Xia and Yuan 2009). Samples for lipidome analysis were taken at 0, 20, and 60 min after the

addition of inhibitors into cells of middle-exponential phase. Comparative studies were carried out between industrial and inhibitor-tolerant strains (SCF, SCP and SCA), respectively. It was found that the addition of inhibitors changed the composition of cell membrane phospholipids, and strains with different capacities in resisting inhibitors showed differences in membrane phospholipid profiles. Statistical analysis of lipidomic data for different strains using Wavelet-Principle Components Analysis (WT-PCA) showed similarities and differences among samples. For each set of data, the score plot divided the samples into intelligible groups, i.e., on the lipidome level samples from tolerant strains, can be discriminated from SC samples. For strain SC, the changes on lipidome level happened as early as 20 min after the addition of the inhibitors. For tolerant strains SCF, SCP, and SCA, the lipidomic changes showed only at 60 min after the addition of certain inhibitors.

Increased unsaturated phospholipids (PLs) for strain SC were found after furfural treatment. The furfural increased the unsaturation level for short-chain PLs at 20 min and for long-chain PLs at 60 min. The increase of unsaturation level for lipids would lead to the increase of the membrane fluidity (Turk et al. 2004), i.e., furfural led to the increase of membrane fluidity for strain SC. When compared with SC, the relative contents of unsaturated PC species for SCF were lower, whereas the contents for saturated PC species were higher. This result indicated that the membrane fluidity for strain SCF was lower than strain SC. The lower membrane fluidity of SCF strain was suggested to offer more potential for increasing tolerance of yeast cells to furfural.

PIs as main biomarkers distinguished the samples of SC from those of SCP. A decrease of short hydrocarbon chain PIs and an increase of long hydrocarbon chain PIs were observed in SCP when compared with SC under control conditions. The higher relative content of long hydrocarbon chain lipids led to lower the membrane fluidity (Russell et al. 1995), meaning, similar to the SCF strain, the SCP strain also possessed lower membrane fluidity than strain SC. The loadings plot showed that PAs were the possible biomarkers for discriminating SC and SCA samples. Under acetic acid stress, SC strain tended to form more long-chain PAs to protect themselves, whereas for SCA, the unsaturation level for PAs decreased after the addition of acetic acid. Therefore, for strain SC, the influence of acetic acid was on the length of the hydrocarbon chains of PAs, whereas for SCA, the influence was on the saturation of the chains of PAs. The changes of both the length and the saturation of hydrocarbon chains of lipids led to the change of the membrane fluidity. These changes were believed to be a signal event other than a structural change of the membrane because the PAs were the minor species of lipids and PAs functioned as precursors for many lipids.

In summary, this study confirmed that the addition of different inhibitors influenced the fluidity of cell membranes, and proper membrane fluidity was of crucial importance for the tolerance against inhibitor stress. It was believed that both the saturation and the length of hydrocarbon chains of phospholipids were important in regulating membrane fluidity.

4 NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is another high throughput technique which can provide valuable measurements on metabolites, with only minimal or no sample preparation steps (Brindle et al. 2002). Molecules of ^{1}H, ^{13}C, and ^{31}P can exist at different energy levels in a strong magnetic field because they possess nuclear spin. Based on this principle, the NMR can detect all proton-bearing compounds simultaneously in a sample, covering most of the compounds, such as sugars, amino acids, organic and fatty acids, amines, esters, ethers, and lipids. Magic-angle spinning NMR can also be used in intact tissues. ^{1}H-NMR can offer a compound's structure information via a unique signal for each chemically distinct hydrogen nucleus, which is believed to be unbiased (Ward et al. 2003). The NMR strategy can also be combined with LC-MS strategy. The LC-NMR-MS approach combined the high-speed NMR screening with the high sensitivity of LC-MS and is thus capable of metabolites separation and identification.

5 Data Mining

In metabolomics, a huge amount of data can be generated from an individual biological sample. It is challenging to deal with the magnanimity data for exploring the underlying molecular mechanism. Suitable data-mining methods could aid analysis and distinguish samples effectively and offer more reliable information about potential biomarkers, which is of great importance for metabolomics. Appropriate multivariate data analysis techniques are applied for the interpretation of the metabolomic results have been well reviewed by Fiehn (2002) and Goodacre et al. (2004). Both unsupervised and supervised pattern recognition methods have been proved to be powerful tools in extracting information from metabolomic data (Lindon et al. 2001).

5.1 Unsupervised

The most widely used unsupervised techniques with metabolomic data include principal components analysis (PCA), hierarchical clustering analysis (HCA), and self-organizing maps (SOMs). The unsupervised techniques are applied to investigate the innate variation in a dataset. The PCA is a commonly used method which can also reduce the number of variables whereas keeping as much information as possible. The principal components are descriptive dimensions that describe the maximum variation within the data.

5.2 Supervised

The most widely used supervised techniques, including discriminant function analysis (DFA), prediction to latent structures through partial least squares (PLS), genetic programming artificial neural network (ANN), and analysis of variance (ANOVA), may be more appropriate, where specific questions are being posed. PLS, the regression extension of PCA, can also be used as a means of data filtering, referred to as orthogonal signal correction (OSC). Variation that is orthogonal to the trend of interest is removed using the PLS. For all the supervised techniques, it is necessary to test the robustness and predictability of the models produced, although the biological function of the metabolites identified may also indicate the success of the particular pattern recognition tool of producing a metabolic profile of a given condition. Most metabolomic datasets contain more variables than samples, thus it is important to reduce the number of variables to obtain uncorrelated features for proper statistical analysis. This can be achieved by using supervised evolutionary algorithms such as genetic algorithms (GAs), genetic programming, or that combined with the secondary algorithm (e.g., DFA or PLS) (Goodacre et al. 2004). The supervised method ANN can get their knowledge by detecting the patterns and relationships in data through training, and can be further used in classification, prediction, and modeling (Takayama et al. 1999).

Efforts have been made to modify traditional pattern recognition methods to maximize the information recovery (Jansen et al. 2004; Scholz et al. 2004). Wavelet transform (WT) has been proved to be an effective tool in capturing the essence of data. In recent years, the applications of the WT in metabolomic research have increased rapidly (Davis et al. 2007; Fonseca et al. 2007). In addition, combinations of the WT method with pattern recognition method such as PCA and ANN have also been reported (Pittner and Kamarthi 1999; Xia et al. 2007).

6 Conclusion and Perspectives

The metabolomic study has contributed significantly to exploring the characteristics of different stages during the industrial fermentation process. The detailed metabolomic analysis combined with multivariate statistical analysis of yeast subjected to stress conditions has identified many important compounds that may be involved in stress responses. These studies have provided important insights into the closely associated metabolic pathways. Moreover, the adaptive mechanisms of yeast were revealed by metabolomics, which provided us further information in selecting stress tolerant strains. Some potential biomarkers identified and quantified in yeast by metabolomics were of great importance in high cell-density fermentation for cell-cell communication and to survive in the stress conditions. These biomarkers could be further validated by correlating with transcriptomics and proteomics data or/and confirming by further analysis of relevant mutants. Further

in-depth metabolomic analysis will aid in identifying signal molecules and regulatory pathways involved in stress responses. Eventually, these efforts will contribute to the identification and selection of novel engineering targets for improved stress tolerance and strain productivity. It is expected that combination of metabolomics with other 'omics' data (Zhang et al. 2010) will provide us a holistic view of how cells respond to stress conditions during the fermentation. Recently, systems metabolic engineering, which was a strategy for strain improvement that based on the results obtained from high throughput systems biology, has just begun to emerge (Askenazi et al. 2003; Stephanopoulos et al. 2004; Park et al. 2008). This strategy not only provides global overview for engineering the metabolic pathways, but also helps us define future directions of process optimization by integration of 'omics' strategies from strain improvement to the final product separation during industrial process.

Acknowledgements The authors are grateful for the financial support from the National Basic Research Program of China ("973" Program: 2007CB714301, 2011CBA00802), and the National Natural Science Foundation of China (Key Program: 20736006, Major International Joint Research Project: 21020102040).

References

Allen J, Davey HM, Broadhurst D, Heald JK, Rowland JJ, Oliver SG, Kell DB (2003) High-throughput classification of yeast mutants for functional genomics via metabolic footprinting. Nat Biotechnol 21:692–696

Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, Blomquist PR, Martinez EJ, Monreal AW, Feibelman TP, Mayorga ME, Maxon ME, Sykes K, Tobin JV, Cordero E, Salama SR, Trueheart J, Royer JC, Madden KT (2003) Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 21:150–156

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357

Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 26:89–105

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science 259:1760–1763

Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligin E, Mosedale DE, Grainger DJ (2002) Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics. Nat Med 8:1439–1444

Caspi R, Foerster H, Fulcher CA, Hopkinson R, Ingraham J, Kaipa P, Krummenacker M, Paley S, Pick J, Rhee SY, Tissier C, Zhang P, Karp PD (2006) MetaCyc: a multiorganism database of metabolic pathways and enzymes. Nucleic Acids Res 34:511–516

Cheng JS, Ding MZ, Tian HC, Yuan YJ (2009a) Inoculation density-dependent responses and pathway shifts in *Saccharomyces cerevisiae*. Proteomics 9:4704–4713

Cheng JS, Zhou X, Ding MZ, Yuan YJ (2009b) Proteomic insights into adaptive responses of *Saccharomyces cerevisiae* to the repeated vacuum fermentation. Appl Microbiol Biotechnol 83:909–923

Corte L, Rellini P, Roscini L, Fatichenti F, Cardinali G (2010) Development of a novel, FTIR (Fourier transform infrared spectroscopy) based, yeast bioassay for toxicity testing and stress response study. Anal Chim Acta 659:258–265

Cowart LA, Shotwell M, Worley ML, Richards AJ, Montefusco DJ, Hannun YA, Lu X (2010) Revealing a signaling role of phytosphingosine-1-phosphate in yeast. Mol Syst Biol 6:349

Cysewski GR, Wilke CR (1977) Rapid ethanol fermentations using vacuum and cell cycle. Biotechnol Bioeng 19:1125–1143

Davis RA, Charlton AJ, Godward J, Jones SA, Harrison M, Wilson JC (2007) Adaptive binning: an improved binning method for metabolomics data using the undecimated wavelet transform. Chemom Intell Lab Syst 85:144–154

de Koning W, van Dam K (1992) A method for the determinations of changes of glycolytic metabolites in yeast on a sub second time scale using extraction at neutral pH. Anal Biochem 204:118–123

Devantier R, Scheithauer B, Villas-Bôas SG, Pedersen S, Olsson L (2005) Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. Biotechnol Bioeng 90:703–714

Ding MZ, Tian HC, Cheng JS, Yuan YJ (2009a) Inoculum size-dependent interactive regulation of metabolism and stress response of *Saccharomyces cerevisiae* revealed by comparative metabolomics. J Biotechnol 144:279–286

Ding MZ, Cheng JS, Xiao WH, Qiao B, Yuan YJ (2009b) Comparative metabolomic analysis on industrial continuous and batch ethanol fermentation processes by GC-TOF/MS. Metabolomics 5:229–238

Ding MZ, Zhou X, Yuan YJ (2010a) Metabolome profiling reveals adaptive evolution of *Saccharomyces cerevisiae* during repeated vacuum fermentations. Metabolomics 6:42–55

Ding MZ, Li BZ, Cheng JS, Yuan YJ (2010b) Metabolome analysis of differential responses of diploid and haploid yeast to ethanol stress. OMICS 14:553–561

Ding MZ, Wang X, Yang Y, Yuan YJ (2011) Comparative metabolic profiling of parental and inhibitors-tolerant yeasts during lignocellulosic ethanol fermentation. Metabolomics. doi:10.1007/s11306-011-0303-6

Fiehn O (2002) Metabolomics-the link between genotypes and phenotypes. Plant Mol Biol 48:155–171

Fonseca ES, Guido RC, Scalassara PR, Maciel CD, Pereira JC (2007) Wavelet time-frequency analysis and least squares support vector machines for the identification of voice disorders. Comput Biol Med 37:571–578

Garcia DE, Baidoo EE, Benke PI, Pingitore F, Tang YJ, Villa S, Keasling JD (2008) Separation and mass spectrometry in microbial metabolomics. Curr Opin Microbiol 11:233–239

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257

Gaspar ML, Aregullin MA, Jesch SA, Nunez LR, Villa-Garcia M, Henry SA (2007) The emergence of yeast lipidomics. Biochim Biophys Acta 1771:241–254

Gonzalez B, Francois J, Renaud M (1997) A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. Yeast 13:1347–1356

Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol 22:245–252

Hajjaj H, Blanc PJ, Goma G, Francois J (1998) Sampling techniques and comparative extraction procedures for quantitative determination of intra- and extracellular metabolites in filamentous fungi. FEMS Microbiol Lett 164:195–200

Halket JM, Waterman D, Przyborowska AM, Patel RK, Fraser PD, Bramley PM (2005) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. J Exp Bot 56:219–243

Han PP, Yuan YJ (2009) Lipidomic analysis reveals activation of phospholipid signaling in mechanotransduction of *Taxus cuspidata* cells in response to shear stress. FASEB J 23:623–630

Hans MA, Heinzle E, Wittmann C (2001) Quantification of intracellular amino acids in batch cultures of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 56:776–779

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA 103:11206–11210

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300–372

Ingram LO, Buttke TM (1984) Effects of alcohols on microorganisms. Adv Microb Physiol 25:253–300

Ivanova PT, Cerda BA, Horn DM, Cohen JS, McLafferty FW, Brown HA (2001) Electrospray ionization mass spectrometry analysis of changes in phospholipids in RBL-2 H3 mastocytoma cells during degranulation. Proc Natl Acad Sci USA 98:7152–7157

Jansen JJ, Hoefsloot HCJ, Boelens HFM, van der Greef J, Smilde AK (2004) Analysis of longitudinal metabolomics data. Bioinformatics 20:2438–2446

Kawai S, Phan TA, Kono E, Harada K, Okai C, Fukusaki E, Murata K (2009) Transcriptional and metabolic response in yeast *Saccharomyces cerevisiae* cells during polyethylene glycol-dependent transformation. J Basic Microbiol 49:73–81

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B, Bergmuller E, Dormann P, Weckwerth W, Gibon Y, Stitt M, Willmitzer L, Fernie AR, Steinhauser D (2005) GMD@CSB.DB: the Golm metabolome database. Bioinformatics 21:1635–1638

Lafaye A, Junot C, Pereira Y, Lagniel G, Tabet JC, Ezan E, Labarre J (2005) Combined proteome and metabolite-profiling analyses reveal surprising insights into yeast sulfur metabolism. J Biol Chem 280:24723–24730

Lei J, Zhao X, Ge X, Bai F (2007) Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. J Biotechnol 131:270–275

Li BZ, Yuan YJ (2010c) Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 86:1915–1924

Li BZ, Cheng JS, Qiao B, Yuan YJ (2010a) Genome-wide transcriptional analysis of *Saccharomyces cerevisiae* during industrial bioethanol fermentation. J Ind Microbiol Biotechnol 37:43–55

Li BZ, Cheng JS, Ding MZ, Yuan YJ (2010b) Transcriptome analysis of differential responses of diploid and haploid yeast to ethanol stress. J Biotechnol 148:194–203

Lin FM, Tang Y, Yuan YJ (2009) Temporal quantitative proteomics of *Saccharomyces cerevisiae* in response to a nonlethal concentration of furfural. Proteomics 9:5471–5483

Lindon JC, Holmes E, Nicholson JK (2001) Pattern recognition methods and applications in biomedical magnetic resonance. Prog Nucl Magn Reson Spectrosc 39:1–40

Liu ZL, Blaschek HP (2010) Biomass conversion inhibitors and in situ detoxification. In: Vertes A, Qureshi N, Yukawa H, Blaschek H (eds) Biomass to biofuels: strategies for global industries. Wiley, West Sussex, pp 233–258

Maharjan RP, Ferenci T (2003) Global metabolite analysis: the influence of extraction methodology on metabolome profiles of *Escherichia coli*. Anal Biochem 313:145–154

Maiorella B, Blanch HW, Wilke CR (1983) By-product inhibition effects of ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol Bioeng 25:103–121

Mannazzu I, Angelozzi D, Budroni M, Farris GA, Gofffini P, Lodi T, Marzona M, Bardi L, Belviso S (2008) Behaviour of *Saccharomyces cerevisiae* wine strains during adaptation to unfavourable conditions of fermentation on synthetic medium: Cell lipid composition, membrane integrity, viability and fermentative activity. Int J Food Microbiol 121:84–91

Martinez A, Rodriguez ME, Wells ML, York SW, Preston JF, Ingram LO (2001) Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol Prog 17:287–293

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686

Nielsen KF, Smedsgaard J, Larsen TO, Lund F, Thrane U, Frisvad JC (2003) Chemical identification of fungi: metabolite profiling and metabolomics. In: Arora DK (ed) Fungal biotechnology in agricultural, food, and environmental applications. Marcel Dekker, New York, pp 19–35

Niessen WMA (1998) Advances in instrumentation in liquid chromatography mass spectrometry and related liquid-introduction techniques. J Chromatogr A 794:407–435

Paley SM, Karp PD (2006) The pathway tools cellular overview diagram and omics viewer. Nucleic Acids Res 34:3771–3778

Palmqvist E, Hahn-Hagerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25–33

Park JH, Lee SY, Kim TY, Kim HU (2008) Application of systems biology for bioprocess development. Trends Biotechnol 26:404–412

Pittner S, Kamarthi SV (1999) Feature extraction from wavelet coefficients for pattern recognition tasks. IEEE Trans Pattern Anal 21:83–88

Plumb RS, Johnson KA, Rainville P, Smith BW, Wilson ID, Castro-Perez JM, Nicholson JK (2006) UPLIC/MSE; a new approach for generating molecular fragment information for biomarker structure elucidation. Rapid Commun Mass Spectrom 20:1989–1994

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J 23:131–142

Russell NJ, Evans RI, ter Steeg PF, Hellemons J, Verheul A, Abee T (1995) Membranes as a target for stress adaptation. Int J Food Microbiol 28:255–261

Scholz M, Gatzek S, Sterling A, Fiehn O, Selbig J (2004) Metabolite fingerprinting: detecting biological features by independent component analysis. Bioinformatics 20:2447–2454

Sen R, Swaminathan T (2004) Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. Biochem Eng J 21:141–148

Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G (2005) METLIN: a metabolite mass spectral database. Ther Drug Monit 27:747–751

Stephanopoulos G, Alper H, Moxley J (2004) Exploiting biological complexity for strain improvement through systems biology. Nat Biotechnol 22:1261–1267

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81:211–223

Takayama K, Fujikawa M, Nagai T (1999) Artificial neural network as a novel method to optimize pharmaceutical formulations. Pharm Res 16:1–6

Tanaka Y, Higashi T, Rakwal R, Wakida S, Iwahashi H (2007) Quantitative analysis of sulfur-related metabolites during cadmium stress response in yeast by capillary electrophoresis-mass spectrometry. J Pharm Biomed Anal 44:608–613

Turk M, Mejanelle L, Sentjurc M, Grimalt JO, Gunde-Cimerman N, Plemenitas A (2004) Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. Extremophiles 8:53–61

Tweeddale H, Notley-Mcrobb L, Ferenci T (1998) Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. J Bacteriol 180:5109–5116

Urbanczyk-Wochniak E, Luedemann A, Kopka J, Selbig J, Roessner-Tunali U, Willmitzer L, Fernie AR (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. EMBO Rep 4:989–993

Van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Rev 32:199–224

Van Hoek P, de Hulster E, Van Dijken JP, Pronk JT (2000) Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. Biotechnol Bioeng 68:517–523

Van Mispelaar VG, Tas AC, Smilde AK, Schoenmakers PJ, van Asten AC (2003) Quantitative analysis of target components by comprehensive two-dimensional gas chromatography. J Chromatogr A 1019:15–29

Villas-Bôas SG, Hojer-Pedersen J, Akesson M, Smedsgaard J, Nielsen J (2005) Global metabolite analysis of yeast: evaluation of sample preparation methods. Yeast 22:1155–1169

Ward JL, Harris C, Lewis J, Beale MH (2003) Assessment of H-1 NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. Phytochemistry 62:949–957

Weeks ME, Sinclair J, Butt A, Chung YL, Worthington JL, Wilkinson CR, Griffiths J, Jones N, Waterfield MD, Timms JF (2006) A parallel proteomic and metabolomic analysis of the hydrogen peroxide- and Sty1p-dependent stress response in *Schizosaccharomyces pombe*. Proteomics 6:2772–2796

Wittmann C, Krömer JO, Kiefer P, Binz T, Heinzle E (2004) Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria. Anal Biochem 327:135–139

Wolf C, Quinn PJ (2008) Lipidomics: practical aspects and applications. Prog Lipid Res 47:15–36

Xia JM, Yuan YJ (2009) Comparative lipidomics of four strains of *Saccharomyces cerevisiae* reveals different responses to furfural, phenol, and acetic acid. J Agr Food Chem 57:99–108

Xia JM, Wu XJ, Yuan YJ (2007) Integration of wavelet transform with PCA and ANN for metabolomics data-mining. Metabolomics 3:531–537

Xia JM, Jones AD, Lau MW, Yuan YJ, Dale BE, Balan V (2010) Comparative lipidomic profiling of xylose-metabolizing *S. cerevisiae* and its parental strain in different media reveals correlations between membrane lipids and fermentation capacity. Biotechnol Bioeng 108:12–21

Yang S, Qiao B, Lu SH, Yuan YJ (2007) Comparative lipidomics analysis of cellular development and apoptosis in two *Taxus* cell lines. Biochim Biophys Acta 1771:600–612

Zhang W, Li F, Nie L (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiology 156:287–301

Zhou X, Zhou J, Tian HC, Yuan YJ (2010) Dynamic lipidomic insights into the adaptive responses of *Saccharomyces cerevisiae* to the repeated vacuum fermentation. OMICS 14:563–574

Automated Systems of Plasmid-Based Functional Proteomics to Improve Microbes for Biofuel Production

Stephen R. Hughes, Tauseef R. Butt, Scott Bartolett, and Steven B. Riedmuller

Contents

1 Introduction 260
2 Plasmid-Based Functional Proteomic Robotic Workcell 261
2.1 Preparation of Plasmid Library on Robotic Workcell 261
2.2 Automated Molecular Biology Protocols for Robotic Workcell 263
2.3 Automated Amino Acid Scanning Mutagenesis Protocol 264
3 Automated Production of Recombinant *S. cerevisiae* Using Selected Genes 266
3.1 Genes Necessary to Engineer Recombinant *S. cerevisiae* to Utilize Biomass 266
3.2 Engineering *S. cerevisiae* with Selected Genes Using SUMO Vector System 266
4 Production of Recombinant *S. cerevisiae* Using Collection of Yeast ORFs 268
4.1 Strategy for Overexpression in *S. cerevisiae* of All ORFs from Yeast Genome 269
4.2 Improved *S. cerevisiae* Overexpressing ORFs from Yeast Genome 269
5 Protein Binding Properties of Recombinant *S. cerevisiae* 271
6 Production of Lipase B in Recombinant *S. cerevisiae* 271
7 Conclusion and Perspectives 273
References 274

S.R. Hughes (✉)
United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Renewable Product Technology Research Unit, Peoria, IL 61604, USA
e-mail: stephen.hughes@ars.usda.gov

T.R. Butt
LifeSensors, Inc., Malvern, PA19355, USA

S. Bartolett
Bartolett Enterprises, Inc., Robotic Software, Robotic Vision, and Robotic Scheduling Units, Bridgewater, NJ 08807, USA

S.B. Riedmuller
Blackland Manufacturing, Inc., Laboratory Workcell Robotics and Integration Design Division, Round Rock, TX 78665, USA

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_11,

Abstract Plasmid-based functional proteomics is an important technology for rapidly obtaining large quantities of protein and determining protein function across an entire genome. It centers on production of full-length cDNA libraries as a source of plasmid-based clones to express the desired proteins in active form to determine their function. Because plasmid libraries are composed of several thousand unique genes, automation of the process is essential. High-throughput platforms that can rapidly clone and express heterologous gene open reading frames (ORFs) in bacteria and yeast and can screen large numbers of expressed proteins for optimized function are important for improving microbial strains for biofuel production. Combined with rapid gene assembly and mutagenesis strategies, gene ORFs can be synthesized, cloned, transformed into yeast strains, and screened to identify those that will give increased ethanol production, allow coproduction of biodiesel, enable use of biomass as a feedstock, and express valuable coproducts. The approach for the past 10 years has been to overexpress proteins that enable microbes to perform functions allowing improved production of biofuels. The next step will be to generate stable strains containing the genes that overexpress these proteins. This will need to be coupled with technologies such as Western blot analysis, high-throughput microscopy, mass spectrometry, gas chromatography, Raman spectroscopy, and microarray analysis to identify critical pathways and metabolites. These techniques adapted to an automated systems biology platform will allow tailoring of microbial strains to use renewable feedstocks for production of biofuels, bioderived chemicals, fertilizers, and other coproducts for profitable and sustainable biorefineries.

1 Introduction

Biochemistry and molecular biology form the basis for all biological and biomedical sciences. A major part of these disciplines is the study of the structure and function of proteins and their diverse biological activities. The production of large amounts of genomic sequence data for a rapidly increasing number of species has changed the approach of protein biochemistry and has given rise to the new discipline of plasmid-based functional proteomics. In addition to classical methods of protein biochemistry, this technology includes high-throughput analyses using 2D gel electrophoresis, mass spectrometry, and combinatorial arrays. Plasmid-based functional proteomics is an important technology for rapidly obtaining large quantities of protein and determining protein function across an entire genome (Lefkovits et al. 2001; Norais et al. 2001; Shaw et al. 2002; Baglioni et al. 2003; Ho et al. 2004; Saad et al. 2005). It also offers possibilities of modifying proteins for optimized functions. This technology centers on the production of full-length cDNA libraries as a source of plasmid-based clones to express the desired protein or proteins in active form for determination of their function. These clones can be used in mutagenesis strategies for optimization of gene open reading frames (ORFs) to develop improved microbial strains and cell lines.

2 Plasmid-Based Functional Proteomic Robotic Workcell

Plasmid-based functional proteomics requires rapid plasmid preparation methods to obtain adequate quantities of high-quality plasmid DNA to conduct all required steps in the process from creation of plasmid libraries to functional testing of expressed proteins. Because the plasmid libraries are composed of several thousand unique genes, automation of the process is essential (Lanio et al. 2000; Holz et al. 2001; Gluck and Wool 2002; Grimm and Kachel 2002; Sebastian et al. 2003; Betton 2004; Finley et al. 2004; Goda et al. 2004; Lorenz 2004; Pajak et al. 2004; Trabbic-Carlson et al. 2004; Kornienko et al. 2005; Lee and Lee 2005). The ideal system would be an automated, integrated programmable workcell capable of producing full-length cDNA libraries, colony picking, isolating plasmid DNA, transforming yeast and bacteria, expressing protein, and performing appropriate functional assays. Such an automated system requires the integration of different equipment and instruments with the desired capabilities. An example of this system is the integrated, plasmid-based proteomic workcell built at USDA NCAUR in Peoria, Illinois. The workcell automates all the required tasks from plasmid library creation through functional testing of the expressed protein(s) for large sets of clones. The robotic workcell (Fig. 1) was designed and built by USDA and Hudson Robotics, Inc. to conduct plasmid-based functional proteomics for optimization of gene ORFs encoding proteins of interest for improved bioenergy applications.

2.1 Preparation of Plasmid Library on Robotic Workcell

The first step in building the robotic workcell was designing and assembling a liquid handler that used a 96-well format for plasmid preparation (Hughes et al. 2005). In addition, a robotic colony-picking component was also integrated onto the workcell platform. To evaluate the operation of the liquid handler and colony picker, a plasmid library of clones expressing mutants of cellulase F (CelF), an endoglucanase enzyme from the anaerobic fungus *Orpinomyces* PC-2 (Chen et al. 2003), was prepared and screened to obtain clones expressing optimized cellulases (Hughes et al. 2006). A multiplexed format was used to reduce the cost of production of recombinant proteins from large-scale plasmid preparations by decreasing the number of wells to be screened and thus decreasing the amount of reagents needed. The multiplex method involves an initial screening of a multiplexed culture that contains a mixture of clones. Individual clones giving rise to improved cellulase mutants are then isolated by picking single colonies from the multiplexed cultures, producing plasmid DNA from the picked colonies, and expressing protein in automated in vitro transcription/translation reactions to identify mutants with improved activity on a plate-based functional assay.

A plasmid library of mutagenized clones of the *celF* gene with targeted variations in the last four codons was constructed by site-directed PCR mutagenesis

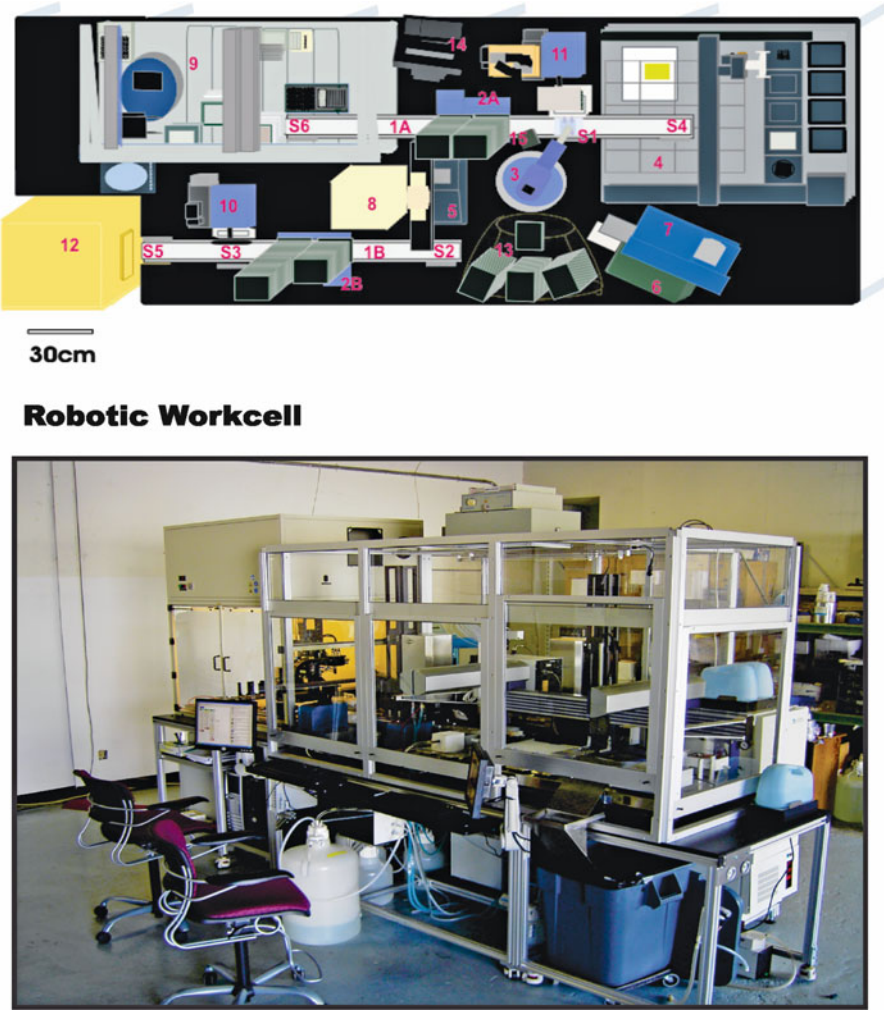

Fig. 1 Schematic and picture of integrated robotic workcell. SoftLink-scheduled, advanced integrated workcell to fully automate high-throughput molecular biology routines and perform high-content screening. *1A* Track 1, *1B* Track 2, *2A* StackLink (Track 1), *2B* StackLink(Track 2), *3* 4-Axis PlateCrane EX, *4* Colony Picker/Arrayer, *5* PCR Thermal Cycler with Autolid, *6* UV/VIS Plate Reader, *7* ABgenePlate Sealer (foil), *8* Plate Sealer (porous tape), *9* Liquid Handler with Centrifuge, *10* Hudson Micro10 Filler, *11* Hudson Plate Aspirator, *12* Automated Incubator, *13* Passive Stackers, *14* Computer and Monitor, *15* Barcode Reader, *S1–S6* StopLink Plate Positions, StopLink Plate

and transformed into *Escherichia coli*. The robotic picker integrated into the workcell was used to inoculate medium in a 96-well deep-well plate, combining eight transformants per well into a multiplexed set, and the plate was incubated on the workcell. Using the liquid handler component of the workcell, plasmids were

prepared from the multiplexed culture and used for in vitro transcription and translation. The expressed recombinant proteins were screened for improved activity and stability in an azo-carboxymethyl cellulose plate assay. Five multiplexed cultures were identified as containing mutants having improved activity. Individual clones were then isolated from the multiplexed cultures using the workcell to inoculate single cultures from stock spread plates, prepare plasmid, produce recombinant protein, and assay for activity, performing all operations on an integrated automated platform. The screening assay and subsequent deconvolution of the multiplexed wells resulted in identification of four improved CelF mutants. The multiplex method using an integrated automated workcell for high-throughput screening in a functional proteomic assay increases the number of clones that can be screened and permits rapid identification of optimized clones.

2.2 Automated Molecular Biology Protocols for Robotic Workcell

Robotic platforms are essential for the production and screening of large numbers of expression-ready plasmid sets used to develop optimized clones and improved microbial strains. An important application of such an automated platform is in the development and screening of optimized genes in high throughput for use in the production of improved commercial yeast strains to convert biomass to ethanol. These strains are being engineered to express genes for hydrolysis and fermentation of cellulose or hemicellulose from plant biomass to ethanol. At the same time, these strains can provide host capability for expression of high-value proteins and peptides, such as a bioinsecticide. Genes for these proteins can be mutagenized and screened in high throughput to optimize the desired functional characteristics. A set of automated molecular biology protocols, including assembly of mutagenized gene sequences, purification of PCR amplicons, ligation of PCR products into vectors, transformation of competent *Escherichia coli*, plating of recovered transformants, and inoculation of cultures for plasmid preparation, was developed for the plasmid-based, integrated robotic workcell.

To demonstrate the application of these protocols, a library of genes encoding variants of a bioinsecticide, lycotoxin-1, from wolf spider (*Lycosa carolinensis*), which is highly effective against insects but not toxic to humans (Yan and Adams 1998), was produced in the pENTR D TOPO vector using PCR mutagenesis in an amino acid scanning strategy to generate a complete set of mutations across the lycotoxin-1 gene. The protocols were used on the integrated, plasmid-based robotic workcell to assemble and purify mutagenized inserts produced by an amino acid scanning mutagenesis strategy, ligate these inserts at high efficiency into a TOPO cloning vector, transform these libraries in high throughput into *E. coli*, inoculate plates for plasmid preparation, and recover the plasmids all in a fully automated fashion. A variation of the multiplex method that was made possible by integration of a robotic colony-picking component onto an automated workcell platform was

used (Hughes et al. 2007). These protocols form the core of a fully automated molecular biology platform, which is essential to allow rapid production of PCR-generated inserts for libraries, whether cDNA libraries or libraries of mutagenized clones, for incorporation into vectors and ultimately, plasmid recovery. A protocol for amino acid scanning mutagenesis was used to generate a complete set of mutations across the lycotoxin-1 gene library (Hughes et al. 2008b). The resulting pENTR D TOPO libraries of assembled or AASM mutagenized products were recombinationally cloned into Gateway-adapted vectors, such as the yeast expression vector pSUMO duo, and used for in vitro and in vivo bacterial expression and for in vivo yeast expression (Butt et al. 2005).

2.3 Automated Amino Acid Scanning Mutagenesis Protocol

Amino acid scanning mutagenesis (AASM) uses the technique of PCR extension to generate mutagenized ORFs of the gene of interest by replacing the original triplet codon for a given amino acid with NNN at the codon position, where N is any of the four possible nucleotide bases. These mutagenized ORFs encode a collection of mutant proteins containing each of the 20 possible amino acids at the given position in the polypeptide chain. The first step in the amino acid scanning mutagenesis protocol (Fig. 2) is an assembly strategy using overlapping oligonucleotides of 50 bp in length to assemble the clone for the gene of interest. A second set of oligonucleotides is produced to assemble a clone with an identical sequence, but the overlap is offset by 25 bp. Once these two clone sets are produced, there is no section of the clone that is not covered by an overlap when introduction of an NNN – NNN – NNN – NNN set of randomization codons is shifted down along both clone set sequences leaving at least 10 bp overlap at the 3′ end of each oligonucleotide. This NNN – NNN – NNN – NNN codon randomization set in one oligonucleotide substituted into each of the two identical assembled clones can potentially give rise to $20^4 = 160{,}000$ variants for all 20 amino acids at each of the positions in the expressed protein corresponding to these four codons. This four-codon substitution yields a manageable number of clones to screen for the average gene length of 1000 nucleotide base pairs. This level of screening can be conducted on most liquid handler-based proteomic workcell robotic platforms on the market, including the unit at NCAUR.

The library of mutagenized genes is expressed using additional automated molecular biology routines, either in vivo or in vitro, on the integrated robotic platform, and the expressed peptides or proteins are screened using various assays, either with intact cells expressing the mutant gene products or with mutants produced by in vitro expression, to identify mutants with optimal characteristics. After optimized clones for these mutants are identified, they are selected and used in a combinatorial algorithm to evaluate all possible combinations of test mutations in the applicable assay.

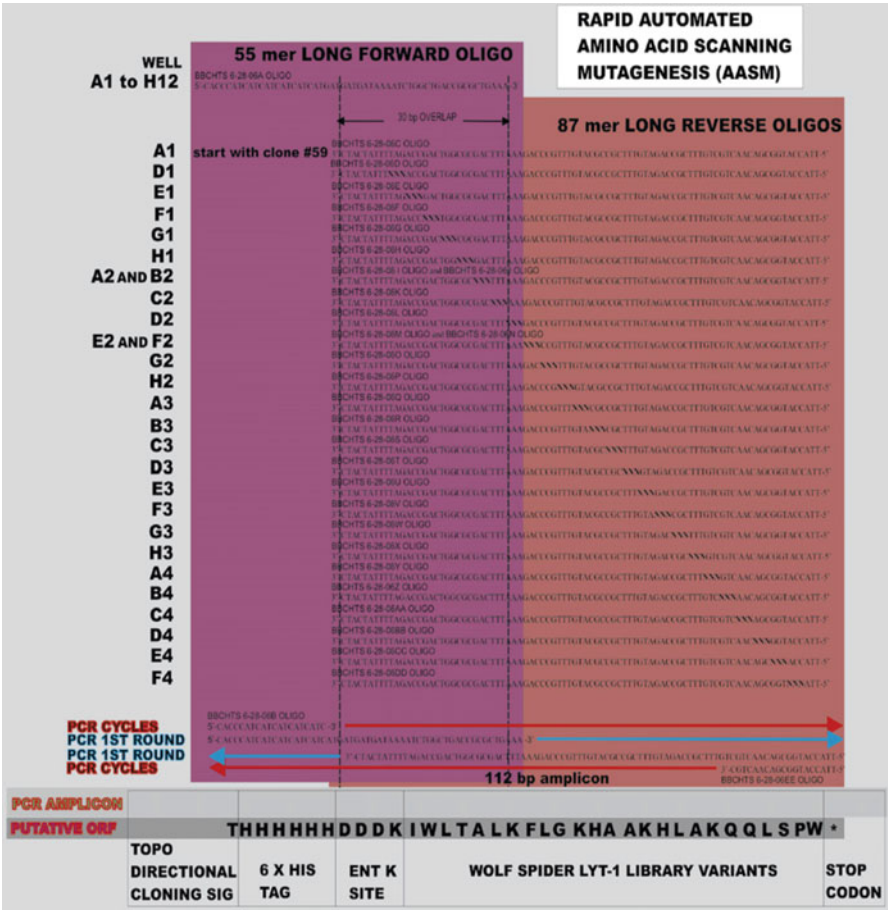

Fig. 2 Amino acid scanning mutagenesis protocol on a plasmid-based, functional proteomic robotic workcell using the Lyt-1 gene sequence that codes for a 25-amino acid protein. Each codon in the Lyt-1 sequence is replaced by NNN, where N is any of the possible nucleotides. Each synthetic mutagenized gene is produced from a 55-nucleotide forward oligonucleotide annealed to an 87-nucleotide reverse oligonucleotide with a 30 bp overlap

Amino acid scanning mutagenesis is much faster and cheaper than randomized mutagenesis because the changes are produced in a systematic fashion along the entire sequence of the clone and mutations are forced to occur at sites that may not change during random mutagenesis. It also has advantages over targeted mutagenesis because the randomized sites in AASM are screened with a functional proteomic assay that selects the randomized oligonucleotides that produce an optimized clone and indicates on which regions to focus. It is also possible to combine the various improved randomized oligonucleotides from AASM to find those combinations that might have a particular synergy to generate a superior optimized open reading frame.

3 Automated Production of Recombinant *S. cerevisiae* Using Selected Genes

Fuel ethanol production from biomass at the industrial level using *Saccharomyces cerevisiae* shows great promise for satisfying future energy demands, but the limited range of materials that can be fermented remains an obstacle to cost-effective bioethanol production (Saha 2003; Farrell et al. 2006). Although several genetically engineered strains of *S. cerevisiae* have been developed that will ferment xylose to ethanol (Sedlak and Ho 2004; Hahn-Hägerdal et al. 2007; Karhumaa et al. 2007; Wisselink et al. 2007), further optimization is needed. It will require the simultaneous expression, at sufficiently high level, of all the enzymes and proteins needed to allow industrial yeast strains to grow efficiently on pentose, as well as hexose, sugars anaerobically. In addition, for cost-effective industrial ethanol production from biomass, it will be necessary to express the enzymes required to saccharify the lignocellulosic feedstocks that are the source of hexose and pentose sugars.

3.1 Genes Necessary to Engineer Recombinant S. cerevisiae *to Utilize Biomass*

Genes considered necessary for complete fermentation of xylose and arabinose, the two major pentose sugar constituents of lignocellulosic biomass, include those encoding xylose isomerase (XI), xylulokinase (XKS), arabinose A, arabinose B, and arabinose D (Karhumaa et al. 2007; Wisselink et al. 2007), which may be obtained from a microorganism naturally capable of fermenting these sugars. In addition, saccharification of lignocellulosic feedstocks requires utilization of hydrolytic enzymes, including cellulases and hemicellulases, after initial chemical pretreatment (Saha et al. 2005; Rudolf et al. 2007). The cost-effectiveness of the ethanol fermentation process could also be enhanced by obtaining high-value coproducts and by-products from the process, such as monomers for polymer production and commercially important proteins and peptides. Genes for these proteins and peptides can be mutagenized, placed in an expression system capable of producing high levels of functional proteins or peptides, and screened in high throughput to optimize desired characteristics.

3.2 Engineering S. cerevisiae *with Selected Genes Using SUMO Vector System*

A three-plasmid yeast expression system utilizing the portable small ubiquitin-like modifier (SUMO) vector set combined with the efficient endogenous yeast protease Ulp1 was developed (Sterner et al. 1999; Wang and Malcolm 1999; Malakhov et al. 2004; Butt et al. 2005) for production of large amounts of soluble functional protein

in *S. cerevisiae* (Sheng and Liao 2002; Li and Hochstrasser 2003). Each vector has a different selectable marker (URA, TRP, or LEU), and the system provides high expression levels of three different proteins simultaneously. This system was integrated into the protocols on an automated, plasmid-based robotic platform to screen engineered strains of *S. cerevisiae* for improved growth on xylose (Fig. 3) (Hughes et al. 2008a).

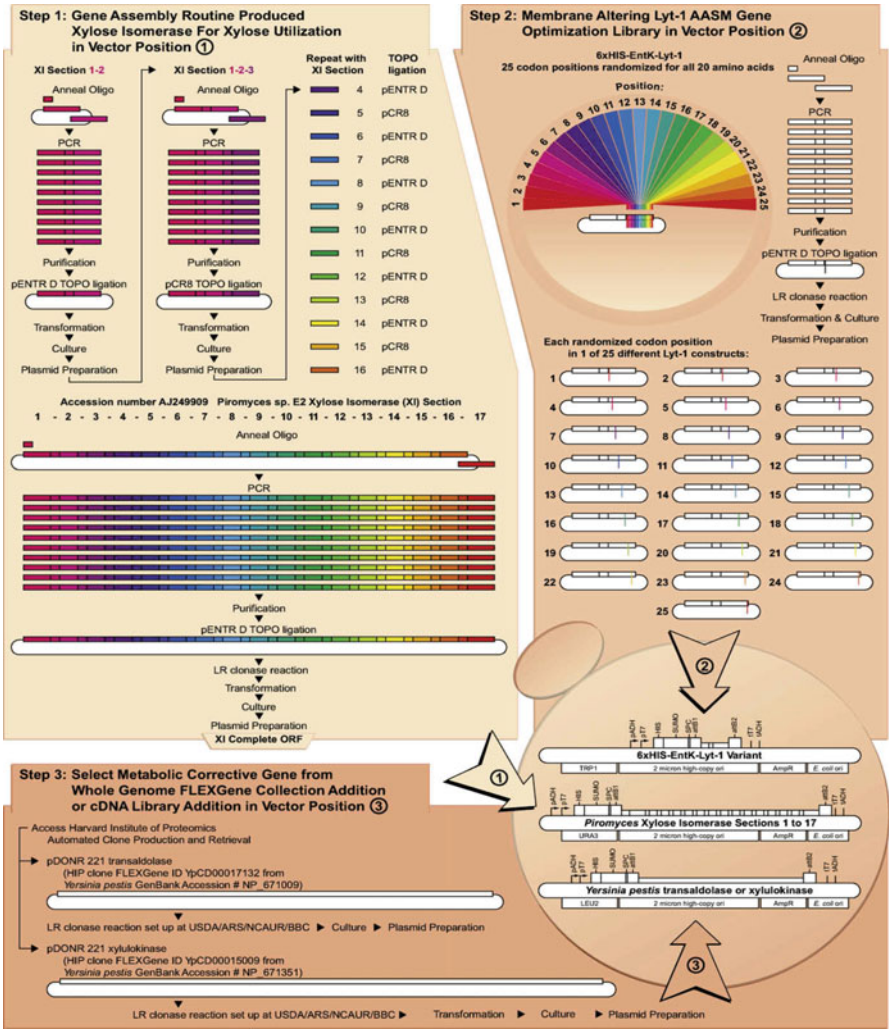

Fig. 3 A three-plasmid yeast expression system utilizing the portable small ubiquitin-like modifier (SUMO) vector set for high expression levels of three different proteins integrated into the protocols on an automated, plasmid-based robotic platform to screen engineered *Saccharomyces cerevisiae* strains for improved growth on xylose. Step 1: Assembly of His-tagged xylose isomerase ORF and cloning into pSUMOduo/URA (vector 1). Step 2: Gene optimization using amino acid scanning mutagenesis (AASM) to randomize lycotoxin-1 at each of 25 positions for all 20 possible amino acids and cloning into pSUMOduo/TRP (vector 2). Step 3: Cloning of additional genes important for xylose utilization into pSUMOduo/LEU (vector 3)

First, a novel PCR assembly strategy was used to clone a *Piromyces* sp. E2 XI gene ORF into the URA-selectable SUMO vector, and the plasmid was placed into the *S. cerevisiae* INVSc1 strain (Hughes et al. 2005), a fast-growing diploid strain ideal for expression (Kuyper et al. 2003, 2004, 2005a), to give the strain designated INVSc1-XI. Second, amino acid scanning mutagenesis was used to generate a library of mutagenized genes (Hughes et al. 2007, 2008b), encoding the bioinsecticidal peptide lycotoxin-1 (Lyt-1), and the library was cloned into the TRP-selectable SUMO vector and placed into INVSc1-XI to give the strain designated INVSc1-XI-Lyt-1. Third, the gene xylulokinase (XKS) of *Yersinia pestis* was moved from pDONR221 (Zuo et al. 2007), cloned into the LEU-selectable SUMO vector, and placed into the INVSc1-XI-Lyt-1 yeast. Yeast strains expressing XI and xylulokinase with or without Lyt-1 showed improved growth on xylose compared to INVSc1-XI yeast. The vectors contain the high-copy 2 μ origin of replication to give a copy number of roughly 20 per yeast cell (Christianson et al. 1992). Expression of XI and XKS is suggested as a means of enabling yeast to metabolize xylose more rapidly through the pentose phosphate pathway (Jin et al. 2003; Kuyper et al. 2004 and 2005a; Van Maris et al. 2007). The SUMO plasmids are particularly well suited for integration with the automated protocols on the robotic platform and complement the PCR assembly and TOPO directional in-frame cloning strategy (Hughes et al. 2005).

This set of plasmids used on the automated platform (Hughes et al. 2005, 2006, 2007) offers the possibilities of expressing pentose-utilization enzymes and commercially important peptides in yeast or introducing other enzymes, such as cellulases, (Den Haan et al. 2007) to produce improved yeast strains for industrial use, and screening the resulting yeast strains in high throughput for those that grow rapidly anaerobically and produce ethanol at sufficiently high levels for industrial application.

4 Production of Recombinant *S. cerevisiae* Using Collection of Yeast ORFs

Engineering the industrial ethanologen *S. cerevisiae* to use pentose sugars from lignocellulosic biomass is critical for commercializing cellulosic fuel ethanol production. One critical need is for robust microbial strains capable of fermenting the more diverse mixture of neutral sugars released by the hydrolysis of lignocellulose. Plant cell wall lignocellulose contains glucose, xylose, arabinose, galactose, and various other sugars. *Saccharomyces* strains are capable of fermenting hexoses; however, they do not ferment the pentose sugars, arabinose or xylose. Engineering strategies to enable *Saccharomyces* to ferment xylose have centered on introducing the needed activities for converting xylose to xylulose using xylose reductase (XR) and xylitol dehydrogenase (XDH). It is significant that engineering approaches to improve pentose-fermenting yeasts have required expression of auxiliary genes to complement activity of XI (Kuyper et al. 2005b). Despite evidence that

overexpression of further genes is required, no systematic screening of the yeast genome has been undertaken to identify the genes that need to be overexpressed for improved xylose fermentation.

4.1 *Strategy for Overexpression in* S. cerevisiae *of All ORFs from Yeast Genome*

A study was performed to evaluate overexpression of each *S. cerevisiae* gene in a strain also expressing XI and determine which of the genes, if any, confer the ability for anaerobic growth on xylose (Hughes et al. 2009a). These genes would be appropriate targets for further improving the fermentation characteristics of xylose-fermenting *Saccharomyces* strains. A high-throughput strategy was implemented to improve anaerobic growth on xylose and rate of ethanol production by evaluating overexpression of each native *S. cerevisiae* gene from a collection of haploid PJ69-4 MATa strains expressing the gene open reading frames (ORFs) mated to a haploid PJ69-4 MATalpha strain expressing the *Piromyces* sp.E2 XI gene. The resulting 6113 diploid strains containing the XI gene and a different yeast gene ORF were screened for growth on xylose in anaerobic plate cultures using an integrated robotic workcell.

4.2 *Improved* S. cerevisiae *Overexpressing ORFs from Yeast Genome*

The study used a collection of *S. cerevisiae* gene open reading frames (ORFs) in pOAD LEU-selectable vectors driven by an alcohol dehydrogenase (*ADH*) promoter in the PJ69-4 MATa *S. cerevisiae* strain (Uetz et al. 2000; Phizicky et al. 2003). Each of these was mated to the haploid PJ69-4 MATalpha *S. cerevisiae* strain containing the *Piromyces* sp.E2 XI gene (Hughes et al. 2008a) expressed from a pDEST32 TRP selectable vector with an *ADH* promoter. To mate and screen the entire collection of gene ORFs, an automated high-throughput strategy incorporating the essential features of the conventional manual process was developed and implemented on an integrated robotic workcell (Fig. 4).

The resulting diploids were selected for anaerobic growth on xylose medium. The effect of xylulokinase (XKS) activity on ethanol production was also evaluated by transforming the diploid strains containing the XI gene and each of the *S. cerevisiae* gene ORFs with pSUMOduo-RGStetHisXKS URA selectable vector. Nine unique strains were isolated that grew anaerobically on xylose selective medium; two were found to no longer grow on glucose; seven were further evaluated for fermentation of alkaline peroxide-pretreated, enzymatically saccharified wheat

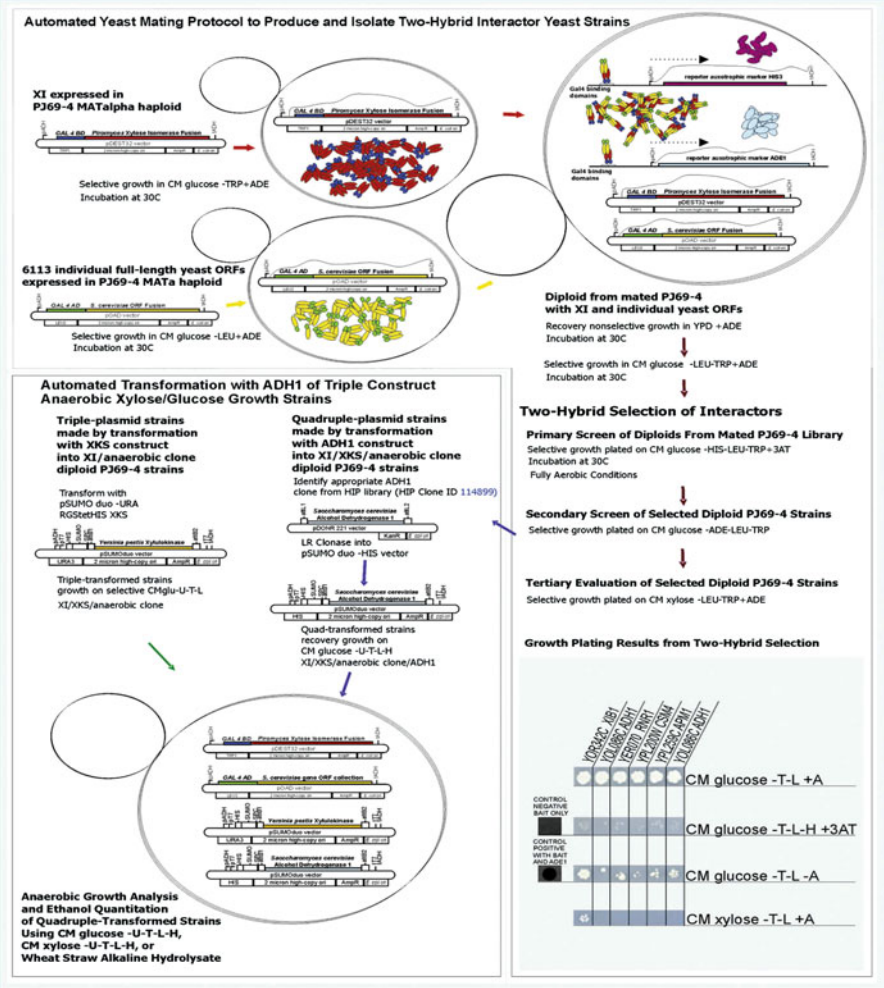

Fig. 4 Automated high-throughput strategy implemented on an integrated robotic workcell to mate and screen a full-genome collection of *S. cerevisiae* gene ORFs for production of recombinant *S. cerevisiae* for improved growth on xylose. Steps required for mating the PJ69-4 MATalpha haploid yeast strain, expressing the *Piromyces* XI gene from the pDEST32 plasmid, to the PJ69-4 MATa haploid yeast strain, expressing one of the collection of yeast genes from the pOAD plasmid

straw hydrolysate. All strains successfully used glucose and xylose, consuming most of the glucose and a small amount of the xylose. Transforming the strains with an additional plasmid expressing the xylulokinase gene did not improve anaerobic growth on xylose but improved glucose use and ethanol production on the hydrolysate, with three of the strains giving maximum ethanol production of 14.0 g/L (Hughes et al. 2009a).

5 Protein Binding Properties of Recombinant *S. cerevisiae*

Attempts to enable the glucose-fermenting industrial yeast *S. cerevisiae* to use pentose sugars from lignocellulosic biomass have focused on introducing the enzymes of the initial stages of xylose metabolism (Van Maris et al. 2006; Hahn-Hägerdal et al. 2007). One approach is to engineer *S. cerevisiae* to express XI, which catalyzes the conversion of xylose to xylulose and does not require redox cofactors. Introduction of a functional XI into *S. cerevisiae* allows slow metabolism of xylose by way of the endogenous enzymes of the nonoxidative part of the pentose phosphate pathway, but this is not sufficient for high rates of anaerobic xylose fermentation. To ensure that flux into and through the pentose phosphate pathway is not a limiting factor, the genes encoding the enzymes involved in this pathway were overexpressed in the strain expressing *Piromyces* sp. E2 XI (Kuyper et al. 2005a). The resulting engineered strain shows a high rate of anaerobic xylose consumption; however, improvement in the rate of ethanol production is still needed for industrial applications (Van Maris et al. 2007).

Very little information is available in the literature on the binding of proteins to XI. The overexpression of endogenous *Piromyces* XI into yeast may expose the fungal enzyme to proteins and possible regulators that are not present in its natural environment. An automated two-hybrid interaction protocol was used (Hughes et al. 2009b) to find yeast genes encoding proteins that bind XI to identify potential targets for improving xylose utilization by *S. cerevisiae*. A pDEST32 vector reengineered for TRP selection and containing the Gal4 binding domain fused with the *Piromyces* sp. E2 XI ORF was used as bait with a library of LEU-selectable pOAD vectors containing the Gal4 activation domain in fusion with members of the *Saccharomyces cerevisiae* genome ORF collection. Binding of a yeast ORF protein to XI activates two chromosomally located reporter genes in a PJ69-4 yeast strain to give selective growth. Five genes, including *ADH*1, were identified in the two-hybrid screen, suggesting that the proteins encoded by these genes bind to XI. These genes are being investigated further for possibly improving xylose utilization by *S. cerevisiae*.

6 Production of Lipase B in Recombinant *S. cerevisiae*

The profitability of ethanol production from lignocellulosic biomass will be improved if high-value coproducts are also generated. Current processes for fuel ethanol production from starch yield substantial amounts of corn oil as a by-product. This corn oil can be used for manufacture of high-quality biodiesel. Corn oil triacyglycerides are converted to fatty acid ethyl esters (biodiesel) and glycerol by transesterification with ethanol. One method of catalyzing this transesterification reaction is with lipase enzymes (Akoh et al. 2007). An integrated biorefinery combining starch ethanol and cellulosic ethanol facilities may become more cost-effective if biodiesel is produced as a coproduct from ethanol and corn oil using

lipase-catalyzed single-step column transesterification (Nielsen et al. 2008) with low-cost lipases expressed in large quantities in a recombinant yeast strain also capable of cellulosic ethanol production. Such a strain is the recently developed recombinant *S. cerevisiae* utilizing the major sugars in biomass hydrolysate, glucose, mannose, arabinose, and xylose, anaerobically to produce ethanol (Hughes et al. 2009a,b). This yeast strain has been engineered to produce ethanol from cellulosic biomass as well as corn starch and could also be engineered to express lipases for biodiesel production from the corn oil by-product of the starch ethanol processes in an integrated biorefinery. Currently, the cost of the enzymatic catalyst is a hurdle compared to the less expensive chemical catalysts, thus the use of recombinant DNA technology to produce large quantities of lipases and the use of immobilized lipases may lower the cost of biodiesel production while reducing downstream processing problems (Villeneuve et al. 2000; Fernández-Lorente et al. 2001; Torres et al. 2003; Akoh et al. 2007; Shibasaki-Kitakawa et al. 2007; Salis et al. 2009).

The scripting of automated protocols and scheduling of PCR assembly steps on the robotic workcell have the potential to be used in an iterative fashion for production of any gene ORF. The *Candida antarctica* lipase B (CALB) gene ORF was produced using a stepwise oligonucleotide PCR assembly strategy followed by TOPO ligation directionally into pENTR D TOPO and LR clonase recombinational cloning into pYES2 DEST 52 vector for expression and evaluation of the lipase enzyme (Fig. 5). The strategy previously described for PCR assembly of the xylose isomerase gene ORF (Hughes et al. 2008a) involved a cloning step after each PCR step. The strategy outlined in Fig. 5 eliminates the subcloning step and assembles the entire ORF in sequential PCR steps so that the process is more rapid and readily adapted for the integrated robotic workcell. The fusion of the C3 variant of the Lyt-1 amphipathic peptide to the lipase potentially facilitates secretion and isolation of the expressed lipase outside the yeast cell for ready availability (Kourie and Shorthouse 2000), in this case, for chemical attachment to a column resin for lipase-catalyzed biodiesel production.

The synthetic *Candida antarctica* lipase B (CALB) gene ORF for expression in yeast was produced, and the lycotoxin-1 (Lyt-1) C3 variant gene ORF was added in-frame with the CALB ORF using the automated PCR assembly and the DNA purification protocol on the integrated robotic workcell. *Saccharomyces cerevisiae* strains expressing CALB protein or CALB Lyt-1 fusion protein were first grown on 2% (w/v) glucose to express enzymes for ethanol production, resulting in production of 9.3 g/L ethanol during fermentation. The carbon source was switched to galactose for *GAL1*-driven expression, and the CALB and CALB Lyt-1 enzymes expressed were tested for fatty acid ethyl ester (biodiesel) production. The expressed CALB enzyme was also immobilized on Sepabeads®, and the activity of the immobilized enzyme in the production of biodiesel was compared to that of nonimmobilized expressed CALB enzyme (Hughes et al. 2011).

The synthetic enzymes were shown to catalyze formation of fatty acid ethyl esters from ethanol and either corn or soybean oil. It was further demonstrated that a one-step-charging resin specifically selected for binding to lipase was capable of

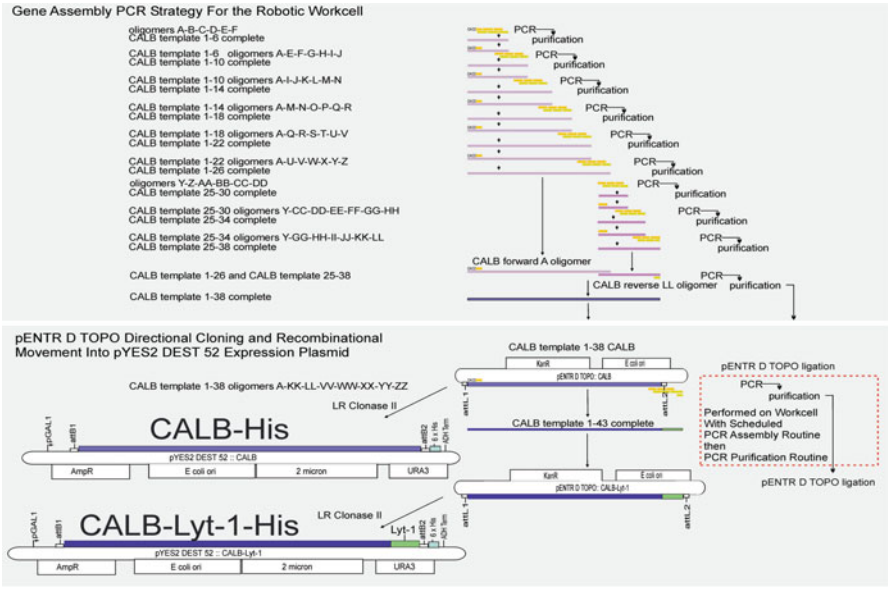

Fig. 5 Diagram of the stepwise assembly strategy used to construct the *Candida antarctica* lipase B (*CALB*) gene ORF expression plasmids. Nine increasingly longer PCR amplicons, 6 at the 5′ end of the CALB sequence and 3 at the 3′ end, were created sequentially from 38 oligonucleotides that included 36 consisting of 50 nucleotides, one consisting of 40 nucleotides, and one consisting of 15 nucleotides for CALB 1–38. Template 1–26 and template 25–38 were combined using PCR to give the CALB 1–38 construct (*top*). PCR assembly and addition of five oligonucleotides containing the Lyt-1 sequence to the 3′ end of CALB 1–38 to give CALB Lyt-1 1–43 were performed on the robotic workcell

covalent attachment of the CALB Lyt-1 enzyme, and that the resin-bound enzyme catalyzed production of biodiesel. High-level expression of lipase in an ethanologenic yeast strain has the potential to increase the profitability of an integrated biorefinery by combining bioethanol production with coproduction of a low-cost biocatalyst that converts corn oil to biodiesel.

7 Conclusion and Perspectives

High-throughput, plasmid-based, functional proteomic platforms that have the capacity to rapidly clone and express heterologous gene ORFs in bacteria and yeast and to screen large numbers of expressed proteins for optimized function are an important technology for improving microbial strains for biofuel production. Combined with rapid gene assembly and mutagenesis strategies on these platforms, gene ORFs can be synthesized, cloned, transformed into yeast strains, and screened to identify those that will give increased ethanol production, allow coproduction of biodiesel, enable use of biomass as a feedstock, and express valuable coproducts.

Algorithms for combining the optimized genes to give the most efficient use of the improved properties are being developed. The overall objective of this technology is to design robust microbes for industrial use. The approach for the past 10 years has been to overexpress proteins to enable microbes to perform functions that will allow more cost-effective production of biofuels. The next step would be to generate stable strains containing the genes that overexpress the multiple proteins that were identified as having improved function. A possible adjunct strategy would be to use antisense technology to attenuate pathways that impede the desired functioning of the microbe to more fully control the expression and function of the cell. This will need to be coupled with technologies, such as Western blot analysis to see the expression and the lack of expression, high-throughput microscopy to look at the morphological changes, and mass spectrometry, gas chromatography, and Raman spectroscopy to identify pathways that have been altered and biochemical metabolites that appear or disappear. In conjunction with microarray analysis, it will be possible to determine the secondary genes that are affected. Adaptation of these technologies to an automated systems biology platform is possible to improve and screen any microbial strain. These techniques will allow tailoring microbial strains to use available feedstocks for production of biofuels, bioderived chemicals, fertilizers, and other coproducts for profitable and sustainable biorefineries.

References

Akoh CC, Chang S-W, Lee G-C, Shaw J-F (2007) Enzymatic approach to biodiesel production. J Agric Food Chem 55(22):8995–9005

Baglioni P, Bini L, Liberatori S, Pallini V, Marri L (2003) Proteome analysis of *Escherichia coli* W3110 expressing an heterologous sigma factor. Proteomics 3(6):1060–1065

Betton JM (2004) High throughput cloning and expression strategies for protein production. Biochimie 86(9–10):601–605

Butt TR, Edavettal SC, Hall JP, Mattern MR (2005) SUMO fusion technology for difficult-to-express proteins. Protein Expression Purif 43:1–9

Chen H, Li X-L, Blum DL, Ximenes EA, Ljungdahl LG (2003) CelF of *Orpinomyces* PC-2 has an intron and encodes a cellulase (CelF), containing a carbohydrate binding module. Appl Biochem Biotechnol 105–108:775–785

Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110(1):119–122

Den Haan R, McBride JE, La Grange DC, Lynd LR, Van Zyl WH (2007) Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. Enzyme Microb Technol 40:1291–1299

Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311(5760):506–508

Fernández-Lorente GR, Fernández-Lafuente R, Palomo JM, Mateo C, Bastida A, Coca J, Haramboure T, Hernández-Justiz O, Terreni M, Guisán JM (2001) Biocatalyst engineering exerts a dramatic effect on selectivity of hydrolysis catalyzed by immobilized ipases in aqueous medium. J Mol Catal B Enzym 11(4–6):649–656

Finley JB, Qiu SH, Luan CH, Luo M (2004) Structural genomics for *Caenorhabditis elegans*: high throughput protein expression analysis. Protein Expression Purif 34(1):49–55

Gluck A, Wool IG (2002) Analysis by systematic deletion of amino acids of the action of the ribotoxin restrictocin. Biochim Biophys Acta 1594(1):115–126

Goda N, Tenno T, Takasu H, Hiroaki H, Shirakawa M (2004) The PRESAT-vector: asymmetric T-vector for high throughput screening of soluble protein domains for structural proteomics. Protein Sci 13(3):652–658

Grimm S, Kachel V (2002) Robotic high throughput assay for isolating apoptosis-inducing genes. Biotechniques 32(3):672–677

Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007) Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 74(5):937–953

Ho K, Xiao Q, Fach EM, Hulmes JD, Bethea D, Opiteck GJ, Lu JY, Kayne PS, Hefta SA (2004) Semi-automated sample preparation for plasma proteomics. J Assoc Lab Autom 9:238–249

Holz C, Lueking A, Bovekamp L, Gutjahr C, Bolotina N, Lehrach H, Cahill DJ (2001) A human cDNA expression library in yeast enriched for open reading frames. Genome Res 11(10):1730–1735

Hughes SR, Riedmuller SB, Mertens JA, Li X-L, Bischoff KM, Cotta MA, Farrelly PJ (2005) Development of a liquid handler component for a plasmid-based functional proteomic robotic workcell. J Assoc Lab Autom 10(5):287–300

Hughes SR, Riedmuller SB, Mertens JA, Li X-L, Bischoff KM, Qureshi N, Cotta MA, Farrelly PJ (2006) High throughput screening of cellulase F mutants from multiplexed plasmid sets using an automated plate assay on a functional proteomic robotic workcell. Proteome Sci 4:10

Hughes SR, Dowd PF, Hector RE, Riedmuller SB, Bartolett S, Mertens JA, Qureshi N, Liu S, Bischoff KM, Li X-L, Jackson JS Jr, Sterner D, Panavas T, Rich JO, Farrelly PJ, Butt TR, Cotta MA (2007) Cost-effective high throughput fully automated construction of a multiplex library of mutagenized open reading frames for an insecticidal peptide using a plasmid-based functional proteomic robotic workcell with improved vacuum system. J Assoc Lab Autom 12(4):202–212

Hughes SR, Sterner DE, Bischoff KM, Hector RE, Dowd PF, Qureshi N, Bang SS, Grynaviski N, Chakrabarty T, Johnson ET, Dien BS, Mertens JA, Caughey RJ, Liu S, Butt TR, LaBaer J, Cotta MA, Rich JO (2008a) Engineered *Saccharomyces cerevisiae* strain for improved xylose utilization with a three-plasmid SUMO yeast expression system. Plasmid 61(1):22–38

Hughes SR, Dowd PF, Hector RE, Panavas T, Sterner DE, Qureshi N, Bischoff KM, Bang SS, Mertens JA, Johnson ET, Li X-L, Jackson JS Jr, Caughey RJ, Riedmuller SB, Bartolett S, Liu S, Rich JO, Farrelly PJ, Butt TR, LaBaer J, Cotta MA (2008b) Lycotoxin-1 insecticidal peptide optimized by amino scanning mutagenesis and expressed as a coproduct in an ethanologenic *Saccharomyces cerevisiae* strain. J Pept Sci 14(9):1039–1050

Hughes SR, Hector RE, Rich JO, Qureshi N, Bischoff KM, Dien BS, Saha BC, Liu S, Cox EJ, Jackson JS Jr, Sterner DE, Butt TR, LaBaer J, Cotta MA (2009a) Automated yeast mating protocol using open reading frames from *Saccharomyces cerevisiae* genome to improve yeast strains for cellulosic ethanol production. J Assoc Lab Autom 14:190–199

Hughes SR, Rich JO, Bischoff KM, Hector RE, Qureshi N, Saha BC, Dien BS, Liu S, Jackson JS, Sterner DE, Butt TR, LaBaer J, Cotta MA (2009b) Automated yeast transformation protocol to engineer *Saccharomyces cerevisiae* strains for improved cellulosic ethanol production with open reading frames that express proteins binding to xylose isomerase identified using a robotic two-hybrid screen. J Assoc Lab Autom 14:200–212

Hughes SR, Moser BR, Harmsen AJ, Bischoff KM, Jones MA, Pinkelman BA, Bang SB, Tasaki K, Doll MD, Qureshi N., Saha BC, Liu S, Jackson JS Jr, Robinson S, Cotta MA, Rich JO, Caimi BA (2011) Production of *Candida antarctica* Lipase B Gene Open Reading Frame Using Automated PCR Gene Assembly Protocol on Robotic Workcell and Expression in an Ethanologenic Yeast for Use As Resin-Bound Biocatalyst in Biodiesel Production. J Lab Autom 16(1):17–37

Jin YS, Ni H, Laplaza JM, Jeffries TW (2003) Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. Appl Environ Microbiol 69(1):495–503

Karhumaa K, Hahn-Hägerdal B, Gorwa-Grausland MF (2005) Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22:359–368

Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund M-F (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Factories 6:5

Kornienko M, Montalvo A, Carpenter BE, Lenard M, Abeywickrema P, Hall DL, Darke PL, Kuo LC (2005) Protein expression plasmids produced rapidly: streamlining cloning protocols and robotic handling. Assay Drug Dev Technol 3(6):661–674

Kourie JI, Shorthouse AA (2000) Properties of cytotoxic peptide-formed ion channels. Am J Physiol Cell Physiol 278:C1063–C1087

Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP, Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4(1):69–78

Kuyper M, Winkler AA, van Dijken JP, Pronk JT (2004) Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res 4(6):655–664

Kuyper M, Hartog MM, Toirkens MJ, Almering MJ, Winkler AA, van Dijken JP, Pronk JT (2005a) Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res 5(4–5):399–409

Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT (2005b) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5:925–934

Lanio T, Jeltsch A, Pingoud A (2000) Automated purification of His6-tagged proteins allows exhaustive screening of libraries generated by random mutagenesis. Biotechniques 29(2):338–342

Lee PS, Lee KH (2005) Engineering HlyA hypersecretion in *Escherichia coli* based on proteomic and microarray analyses. Biotechnol Bioeng 89(2):195–205

Lefkovits I, Kettman JR, Frey JR (2001) Proteomic analysis of rare molecular species of translated polypeptides from a mouse fetal thymus cDNA library. Proteomics 1(4):560–573

Li S-J, Hochstrasser M (2003) The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. J Cell Biol 160(7):1069–1081

Lorenz MGO (2004) Liquid-handling robotic workstations for functional genomics. J Assoc Lab Autom 9:262–267

Malakhov MP, Mattern MR, Malakhova OA, Drinker M, Weeks SD, Butt TR (2004) SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. J Struc Func Genomics 5(1–2):75–86

Nielsen PM, Brask J, Fjerbaek L (2008) Enzymatic biodiesel production: technical and economical considerations. Eur J Lipid Sci Technol 110(8):692–700

Norais N, Nogarotto R, Lacobini ET, Garaguso I, Grifantini R, Gauli G, Grandi G (2001) Combined automated PCR cloning, in vitro transcription/translation and two-dimensional electrophoresis for bacterial proteome analysis. Proteomics 1(11):1378–1389

Pajak L, Zhang R, Pittman C, Roby K, Boyer S (2004) Automated genomic and proteomic applications on the Biomek® NX laboratory automation workstation. J Assoc Lab Autom 9:177–184

Phizicky E, Bastiaens PIH, Zhu H, Snyder M, Fields S (2003) Protein analysis on a proteomic scale. Nature 422:208–215

Rudolf A, Baudel H, Zacchi G, Hahn-Hägerdal B, Lidén G (2007) Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. Biotechnol Bioeng 99(4):783–790

Saad MM, Kobayashi H, Marie C, Brown IR, Mansfield JW, Broughton WJ, Deakin WJ (2005) NopB, a type III secreted protein of *Rhizobium* sp. strain NGR234, is associated with pilus-like surface appendages. J Bacteriol 187(3):1173–1181

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30(5):279–291

Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol Prog 21(3):816–822

Salis A, Bhattacharyya MS, Monduzzi M, Solinas V (2009) Role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase used for biodiesel synthesis. J Mol Catal B Enzym 57:262–269

Sebastian P, Wallwitz J, Schmidt S (2003) Semi automated production of a set of different recombinant GST-streptag fusion proteins. J Chromatogr B Anal Technol Biomed Life Sci 786(1–2):343–355

Sedlak M, Ho NWY (2004) Production of ethanol from cellulosic biomass hydrolysates using genetically engineered *Saccharomyces* yeast capable of cofermenting glucose and xylose. Appl Biochem Biotechnol 114(1–3):403–416

Shaw AC, Gevaert K, Demol H, Hoorelbeke B, Vandekerckhove J, Larsen MR, Roepstorff P, Holm A, Christiansen G, Birkelund S (2002) Comparative proteome analysis of *Chlamydia trachomatis* serovar A, D and L2. Proteomics 2(2):164–186

Sheng W, Liao X (2002) Solution structure of a yeast ubiquitin-like protein Smt3: the role of structurally less defined sequences in protein-protein recognitions. Protein Sci 11:1482–1491

Shibasaki-Kitakawa N, Honda H, Kuribayashi H, Toda T, Fukumura T, Yonemoto T (2007) Biodiesel production using anionic ion-exchange resin as heterogeneous catalyst. Bioresour Technol 98(2):416–421

Sterner DE, Grant PA, Roberts SM, Duggan LJ, Belotserkovskaya R, Pacella LA, Winston F, Workman JL, Berger SL (1999) Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. Mol Cell Biol 19(1):86–98

Torres R, Mateo C, Fernández-Lorente G, Ortiz C, Fuentes M, Palomo JM, Guisán JM, Fernández-Lafuente R (2003) A novel heterofunctional epoxy-amino Sepabeads for a new enzyme immobilization protocol: immobilization-stabilization of beta-galactosidase from *Aspergillus oryzae*. Biotechnol Prog 19(3):1056–1060

Trabbic-Carlson K, Liu L, Kim B, Chilkoti A (2004) Expression and purification of recombinant proteins from *Escherichia coli*: comparison of an elastin-like polypeptide fusion with an oligohistidine fusion. Protein Sci 13(12):3274–3284

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403:623–627

Van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Leeuwenhoek 90(4):391–418

Van Maris AJA, Winkler AA, Kuyper M, de Laat WTAM, van Dijken JP, Pronk JT (2007) Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. Adv Biochem Eng Biotechnol 108:179–204

Villeneuve P, Muderhwa JM, Graille J, Haas MJ (2000) Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. J Mol Catal B Enzym 9(4–6):113–148

Wang W, Malcolm BA (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. Biotechniques 26(4):680–682

Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, van Dijken JP, Pronk JT, van Maris AJA (2007) Engineering of saccharomyces cerevisiae for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol 73(15):4881–4891

Yan L, Adams ME (1998) Lycotoxins, antimicrobial peptides from venom of the wolf spider *Lycosa carolinensis*. J Biol Chem 273(4):2059–2066

Zuo D, Mohr SE, Hu Y, Taycher E, Rolfs A, Kramer J, Williamson J, LaBaer J (2007) PlasmID: a centralized repository for plasmid clone information and distribution. Nucleic Acids Res 35 (Database issue):D680–D684

Unification of Gene Expression Data for Comparable Analyses Under Stress Conditions

Z. Lewis Liu

Contents

1 Introduction 280
2 The Concept of the External RNA Control 281
3 Microarray Assay 283
3.1 Microarray Design and Fabrication with Quality Controls 283
3.2 The Universal External RNA Controls 283
3.3 Quality Control for Labeled Probes Using sGel 286
3.4 Data Acquisition and Normalization 287
4 Real-Time qRT-PCR Assay 288
4.1 Calibration of Linear Dynamic Range 288
4.2 Robust PCR CT Reference 290
4.3 The Master Equation of Standard Curves 290
4.4 PCR Amplification Efficiency 292
5 Unification of Gene Expression Data for Comparable Analyses 293
5.1 Microarray 293
5.2 Real-Time qRT-PCR and PCR Array 294
5.3 Comparison Across Different Assay Platforms 294
5.4 Troubleshooting 295
6 Conclusion and Perspectives 296
References 297

Abstract Gene expression is a fundamental biological process in which genotypes rise to phenotypes. As a quantitative measurement, expression of a gene is commonly examined by mRNA abundance that varies in response to different conditions and environmental stimuli. High throughput quantitative measurements of gene expression data have difficulties of reproducibility and comparability due to a lack of standard mRNA quantification references. Efforts have been made to

Z.L. Liu (✉)
National Center for Agricultural Utilization Research, USDA-ARS, 1815 N. University Street, Peoria, IL 61604, USA
e-mail: ZLewis.Liu@ars.usda.gov

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7_12,

safeguard data fidelity, yet generating quality expression data of inherent value remains a challenge. This not only affects unbiased data assessment and clinical applications but also damages establishing invaluable database resources for the larger scientific community. Unification of multi-source gene expression data is necessary for comparable and comprehensive analyses to gain insight into complex gene interactions and regulatory networks of life events using more integrated approaches of bioinformatics, computational biology and systems biology. Development and application of commonly accepted quantification references to generate comparable expression data are urgently needed. This chapter provides basics and application aspects for comparative gene expression analyses using microbial examples under stress conditions.

1 Introduction

Rapid advances in genome sequencing have led to the widespread applications of global transcriptome and gene expression analyses using microarray and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in many fields of biology including microbial stress tolerance. Gene expression as measured by mRNA abundance varies in response to different conditions and environmental stimuli. It is a useful means to characterize phenotypes of microbial response to various stress conditions and provides significant insight into gene regulatory mechanisms of complex interactions and networks in biological events. The use of high throughput assay technologies has flourished for more than a decade; yet accurate data acquisition and reproduction of quality expression data remain challenging. A lack of standard quantification references for normalization of gene expression data has been recognized as a key issue that hinders reproducibility and comparability of expression data (Klein 2002; Huggett et al. 2005; ERCC 2005a).

For conventional practice, housekeeping genes have been applied as an internal reference for data normalization and analysis since the technology first appeared (PE Applied Biosystems 1997; Collins et al. 1998). Performance of housekeeping genes varies with the background of the host genome, experimental conditions, sequence preference, and even labeling dyes applied. Such variation adds complexity to the multiphase sources of variations attributed to the biology and the technology. When housekeeping genes are used, a great care must be taken in gene selection and data interpretations under different conditions. Because of the variability of housekeeping genes in response to different conditions, there is no commonly accepted housekeeping gene reference available (Mohsenzadeh et al. 1998; Tricarico et al. 2002; Baeber et al. 2005; ERCC 2005a; b; Goldsworthy et al. 1993). Acquisition and processing of expression data is at the discretion of individual researchers, and thus data obtained are often not reproducible nor comparable. The variety of data handling options for qRT-PCR is even more complex although the assay platform is considered less variable. The reproducibility of expression data has been an ever increasing concern of the research and science community. To safeguard the quality

of data, minimum requirements for reporting high throughput expression data have been established (Brazma et al. 2001; Wong and Medrano 2005; VanGuilder et al. 2008; Bustin et al. 2009). Currently, most data sets obtained from different experimental conditions or across different studies are not comparable. This not only affects capabilities of individual investigations under different experimental conditions but also limits the utilization of a vast amount of expression data generated by researchers. For example, a large amount of expression data available in the Saccharomyces Genome Database (Fisk et al. 2006) obtained under diversified conditions have limited potential to be fully exploited. Similar situations exist within publically available resources, including the NCBI database.

For microbial stress tolerance studies, a reliable quantification reference of quality control for gene expression data is vital since the expression levels vary significantly under different physiological conditions. Comprehensive data analyses from varied experimental sets and conditions are necessary, and unification of a large amount of expression data is often needed. Therefore, it is essential to generate reproducible data for comparative analyses (Liu 2010). An ideal reference gene for this purpose should be robust and independent of experimental conditions. Ribosome RNA genes are conserved and 18S rDNA, for example, is commonly used for yeast as a reference gene. Although it is less susceptible to environmental stimuli, its variations were observed under clinic conditions (Tricarico et al. 2002). In addition, the relative more abundance and high levels of sensitivity in PCR often cause over estimation and false signals for quantitative analysis of most target genes. Therefore, 18S rDNA is not recommended as a normalization reference (Liu and Slininger 2007). Instead, a universal external RNA control system is suggested to be used as a refernce.

2 The Concept of the External RNA Control

In contrast to the endogenous gene control references, carefully selected external nucleic acids that have no sequence similarities can avoid potential cross-reactions with a host-genome background. It is assumed that the external mRNA control has the same amplification efficiency (see descriptions in a later section) with the host genome, especially for qRT-PCR assays. A variety of external RNA controls has been developed for different types of applications (Smith et al. 2003; Cronin et al. 2004; Novoradovskaya et al. 2004; Huggett et al. 2005; Kanno et al. 2006; Liu and Slininger 2007; Bower et al. 2007; Kakuhata et al. 2007; Ellefsen et al. 2008). The universal RNA controls for microbial applications provided the first quality control system that can be applied to different platforms of microarray and qRT-PCR (Liu and Slininger 2007). As demonstrated for use with yeast and a bacterium, this quality control system is suitable for microbial gene expression analyses, including filamentous fungi (Liu et al. 2009a). It is suitable for multiple reaction applications, such as qRT-PCR arrays, under different experimental conditions (Liu et al. 2009a, b; Ma and Liu 2010). Unification of gene expression data for comparable analyses

is possible when the quantification references are applied in proper assay steps (Fig. 1). It also allows comparisons of data obtained from different platforms of microarray and qRT-PCR when applied within valid detection of linear dynamic ranges (see describtion in a later section) shared by the two assay platforms. The current protocols can be readily adapted for laboratory and high throughput assays without additional instrument and software investments.

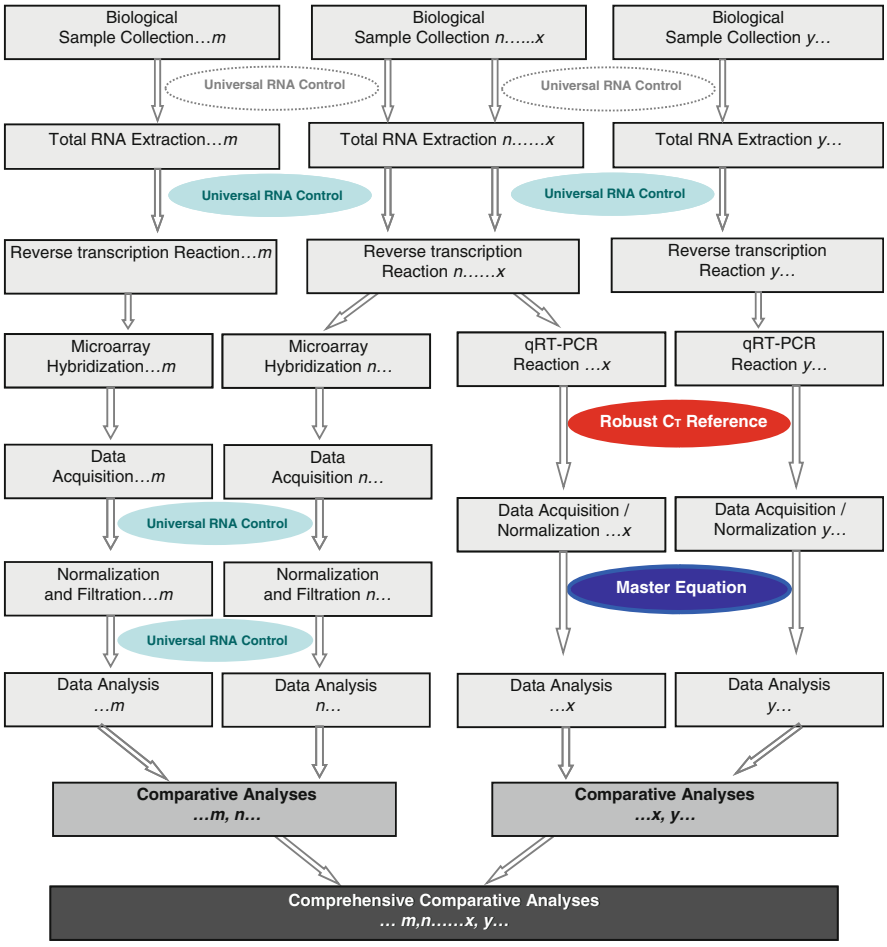

Fig. 1 A schematic diagram showing applications of defined quantification reference in microbial gene expression assays using microarray and real time qRT-PCR. Application steps are highlighted with universal RNA controls, a PCR cycle threshold (CT) reference, and a master equation. The non-shaded universal RNA control step prior to total RNA extraction is optional. The robust references can be used for comparative data analysis within a specific assay platform intensively as well as for comparisons of quantification data falling within a valid overlapping linear detection range from 10 to 1,000 pg between microarray and qRT-PCR platforms (Liu, 2010)

3 Microarray Assay

Numerous platforms of microarray assays are available. Discussions in this section use an example of two-color "homemade" spotted microarray.

3.1 Microarray Design and Fabrication with Quality Controls

Probes of the quality control genes for microarray need to be incorporated into the microarray printing design. A DNA microarray is generally arranged in numerous blocks, each containing a group of target gene probes. Using the *Saccharomyces cerevisiae* genome as an example, its 6,388 genes can be distributed into 16 blocks for the entire genome (Fig. 2a). At least two replicated genome printings should be made and in this example, three replications. For two-color DNA oligo microarray, a repeated laser scanning is often applied during data acquisition to balance signal intensities of Cy3 and Cy5. Since Cy5 is more sensitive than Cy3 and imbalanced dye breach happens due to exposure of the laser scanning that influences data reliability. A simple mini-array containing a set of control reference genes placed on the top of the target array is helpful in resolving this problem (Fig. 2a). The target array consists of six universal RNA controls (to be discussed in the next section) and two background controls such as a printing buffer and a polyA used in hybridization step (Fig. 2b). Each probe in the mini-array can be arranged in 16–32 or more replications. During the hybridization, the mRNA of each control can be spiked-in at desirable doses, for example, a calibration dose ranged from 10 to 7,000 pg, as expected detection reference (see Liu and Slininger 2007 for detailed descriptions). As presented in this example, three controls were used for linear calibration, one for three-fold differential expression by Cy3, one for three-fold by Cy5, and one for negative control (Fig. 2c). During the pre-scan, such as using the GenePix 4,000B scanner, the laser PMT gain can be adjusted repeatedly using the signal intensities of these references. Then the optimized laser power can be applied for scanning the target array once and the intact signal intensities of both channels are preserved. For the target array, the controls are embedded in each block with duplicated prints (Fig. 2d). The total number of replications of each reference gene on a target array can reach 64 and 96 for two and three genome replications, respectively. These references are necessary for evaluation of the quality of the assay and to identify differential expressions (Fig. 2e) (see a later section for detailed descriptions).

3.2 The Universal External RNA Controls

Based on sequence blast searches, six genes showing no homologous sequence similarities with the microbial sequence database were selected as universal RNA

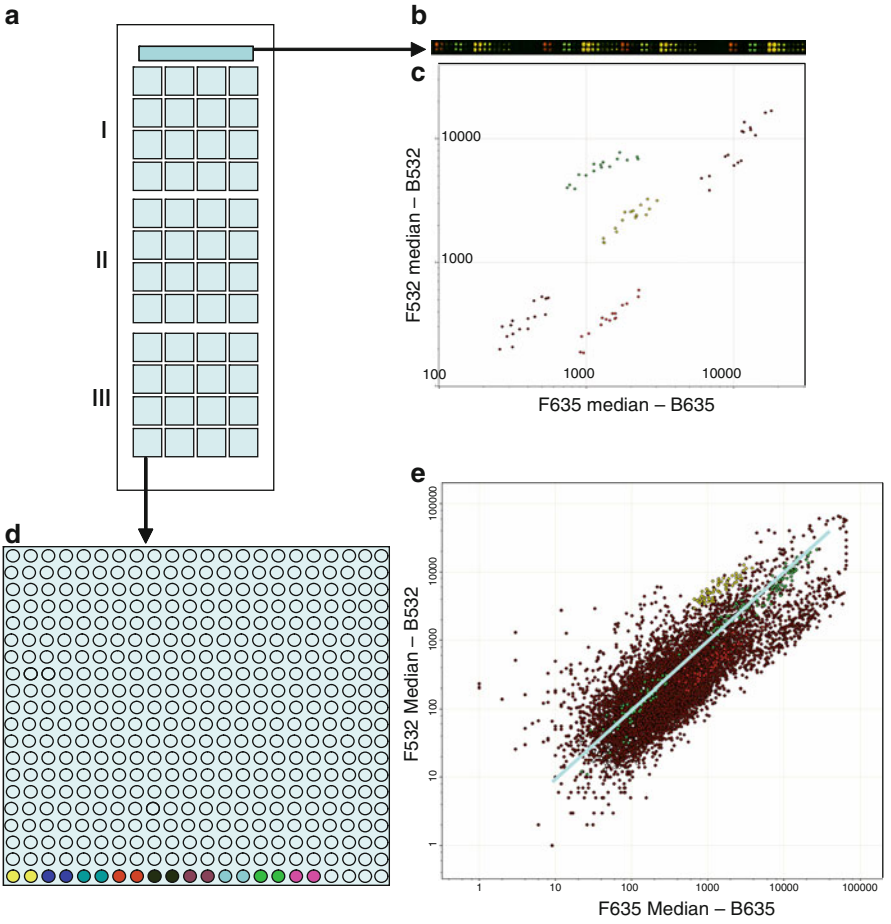

Fig. 2 A schematic diagram showing a microarray slide design with quality control measurements and the functionality of the universal RNA controls. A pre-scan reference mini-array is placed on the top of three replicated target genome arrays on a microarray slide (**a**). the mini-array (**b**) consists of 16 replications each of the six universal external RNA controls and negative background controls of polyA and buffer. A scatter plot of the control genes (**c**) indicating 16 replications of 7,000 pg (*CtrlGm_4, upper right, brown spots*), 1,000 pg (*CtrlGm_5, yellow*) and 50 pg RNA input (*CtrlGm_6, lower left, brown spots*) in linear range, and as well as a threefold differential expression reference towards Cy3 (*CtrlBt_3, green*) or Cy5 (*CtrlBt_1, red*) channel, respectively. Signal intensities are presented by mean values of foreground median pixel subtracted by the background at wavelength 635 nm for Cy5 (F635 median – B635) and at 532 nm for Cy3 (F532 median – B532), respectively. Each target array consists of 16 blocks with the universal RNA controls and background controls embedded in each block (**d**). Functional performance of the controls in microarray assay (**e**) showing many genes were differentially expressed under the stress condition in a lignocellulose derived inhibitor-stress challenged yeast microarray experiment. In contrast, calibration controls, CtrlGm_4, 5 and 6 showed consistent expression as a normalization reference (green spots and central line) and differential expression reference gene CtrlBt_1 (*Cy5, red*) and CtrlBt_3 (*Cy3, yellow*) consistently showed three-fold expression toward each dye channel, respectively

references for microbial gene expression quality controls (ERCC 2005a; Liu and Slininger 2007). Three external reference genes, *ACTB* (beta-actin), *B2M* (beta-2-microglobulin) and *PGK1* (phosphoglycerate kinase 1) from Bovine (*Bos taurus*) are designated as CtrlBt_1, 2, and 3, respectively; and three external genes *CAB* (chlorophyll A-B binding protein of LHCI type III precursor), *MSG* (major latex protein) and *RBS1* (ribulose bisphosphate carboxylase small chain 1 precursor) from soybean (*Glycine max*) designated as CtrlGm_4, 5, and 6, respectively. The complete sequence information for these genes is available at NCBI GenBank under accession AW464237, AW465604, AW465431, BE190670, AJ239127, and AI495218, respectively. The 70-mer oligo control probes can be synthesized using the defined sequences with an aminoC6 modification at the 5′-end and ready to use (Table 1). Detailed descriptions of the gene in vitro transcription and additional primers are available elsewhere (Liu and Slininger 2007).

In order to detect the 70-mer probes of the controls printed on a microarray slide, the control genes are labeled along with a host genome during reverse transcription followed by hybridization. The control mix containing desirable concentrations for each gene is spiked into a total RNA followed by a reverse transcription reaction as described (Liu and Slininger 2007). For two-color microarray experiments,

Table 1 Universal RNA controls of DNA 70-mer oligonucleotide probes for microarray and primers and TaqMan probes for qRT-PCR designed for microbial gene expression analysis

Gene	ID	Sequence 5′-3′	Amplicon (bp)
For Microarray use			
ACTB	CtrlBt_1	(AminoC6) GAGCTACGAGC TTCCTGACGGGC AGGTCATCACCAT CGGCAATGAGCG GTTCCGCTGC CCTGAGGC TCTC	–
B2M	CtrlBt_2	(AminoC6) GTCCTCCAAAG ATTCAAGTGTACT CAAGACACCCACC AGAAGATGGAAA GCCAAATTACCT GAACTGCTA	–
PGK1	CtrlBt_3	(AminoC6) ATGAGGTGGTGAA AGCCACTTCCAG GGGCTGCATCA CCATCATAGGTG GTGGAGACACTGC TACTTGCTG	–
RBS1	CtrlGm_4	(AminoC6) GAAGACC AACAATG ACATTACCTC CATTGCTAGC AACGGTGGAAGA GTGCAATGCA TGCAGGTGTG GCCA	–
CAB	CtrlGm_5	(AminoC6) CTAGCATATGG TGAGATAATCA ATGGTCGTTAT GCAATGTTGGG TGCAGTTGGTGCAA TAGCACCTGAAA	–
MSG	CtrlGm_6	(AminoC6) ACACTGTTGA GACCTTAAAGG AGAGAGTTGA TTTTGATGATG AAAACAAGAAGA TAACCTACA CCATATT	–
For qRT-PCR use			
MSG	MSG_left	GATGAGCACAGCCTTGTGAA	112
	MSG_pT	TET-TGAGAAGGTGGATCACACTG-TAMRA	
	MSG_right	CCTCCACGTTCTTGGTGAGT	
CAB	CAB_left	AGACAGCACTCCCATGGTTC	109
	CAB_pV	VIC-TTCCCACCTGCAGGAACCTA-TAMRA	
	CAB_right	AATCCCATCAGTGCCATCTC	
RBS1	RBS1_left	GCTTGGAATTCGAGTTGGAG	123
	RBS1_pF	6FAM-TACCGTGAGCACAACAGGTC-TAMRA	
	RBS1_right	GAGAAGCATCAGTGCAACCA	
ACTB	ACTB_left	GCTCTCTTCCAGCCTTCCTT	104
	ACTB_pV	VIC-CATTCACGAAACTACCTTCA-TAMRA	
	ACTB_right	TAGAGGTCCTTGCGGATGTC	
B2M	B2M_left	AGCGTCCTCCAAAGATTCAA	127
	B2M_right	TCCCCATTCTTCAGCAAATC	

the spiking-in controls are prepared as a mix of known concentrations of RNA transcripts to be used in each dye-labeling reaction. For example, spiking-in mix I, CtrlGm_high, can be made for regular or high abundance mRNA analysis, consisting of CtrlGm_4, 5, and 6 at concentrations of 7,000, 1,000, and 50 pg/μl for each control gene. Spiking-in mix II, CtrlGm_low, is prepared for lower abundance of mRNA analysis, consisting of the three genes each at a concentration of 1,000, 100 and 10 pg/μl, respectively. One of the control mixes is added to each dye-labeling reaction and serves as a calibration standard. In addition, a mix for a three-fold differential expression reference for each dye, Cy3 and Cy5, is prepared separately. In this example, CtrlBt_Cy3 is prepared consisting of CtrlBt_1 and 3 at concentrations of 500 and 6,000 pg/μl, respectively, for the Cy3 labeling reaction. CtrlBt_Cy5 consists of 1,500 and 2,000 pg/μl for CtrlBt_1, and 3, respectively, for the Cy5 labeling reaction. Accordingly, RNA labeling reactions with CtrlBt_Cy3 and CtrlBt_Cy5 are expected to have CtrlBt_1 expressed three-fold greater for Cy5 than Cy3, and CtrlBt_3, three-fold greater for Cy3 than Cy5. CtrlBt_2 serves as a negative control of DNA sequence background with its probe printed on a microarray slide, but no RNA transcript is spiked in the labeling reaction.

3.3 Quality Control for Labeled Probes Using sGel

Hybridization probes used for microarray assay are cDNA populations reverse transcribed from varied sources of total RNA. The quality of RNA and numerous factors affect the length of cDNA and the probe-labeling efficiency for the complete cDNA populations. Therefore, examination of the equivalent quality of cDNA populations before hybridization is necessary for unbiased assessment of differential expressions. A Nano-Drop Spectrophotometer-based measurement is excellent for quantification of the labeled nucleic acid, but unable to access the quality of the cDNA in terms of varied lengths of transcripts. Several gel-based methods are available to aid evaluation of cDNA labeling quality (Lage et al. 2002; Liu and Slininger 2007). A simple and quick method of running a mini-gel on a slide, sGel, is recommended for routine laboratory assays (Fig. 3) (Liu and Slininger 2007). Using this method, a microscopic slide is coated with a thin layer of 1% agarose gel in 1x TBE buffer. One μl of purified labeling reaction for Cy3 or Cy5 is mixed with 2 μl of 50% glycerin separately and loaded into an open well on the slide gel separately. The horizontal electrophoresis is carried out at 120v for 30 min, avoiding exposure of light to protect Cy-dye from bleaching. Upon completion of gel electrophoresis, the gel is dried on the slide at 65°C and then scanned using a scanner, for example, GenePix 4,000B. Comparisons of the cDNA-labeling efficiency then can be made between Cy3- and Cy5-labeled probes. A four-lane sGel can be conveniently prepared and run on a microscope slide with high resolution. A Cy dye-labeled probe can be detected to have high levels of the labeled concentrations but not necessarily represent a complete cDNA population, especially for large cDNA species. Therefore, probes showing sufficient Cy

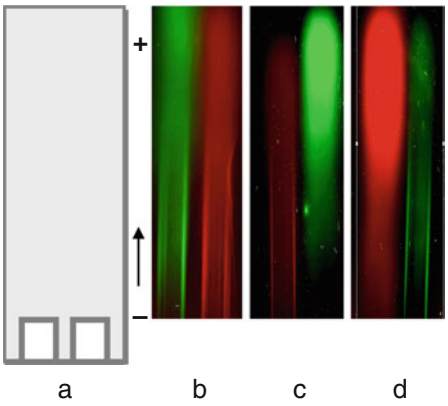

Fig. 3 Quality control assessment of labeled RNA probe for microarray hybridization experiments. A set-up of slide gel electrophoresis with two or four lanes (**a**) and scanned images of the slide gels demonstrating varied cDNA lengths of Cy3- (*green*) or Cy5-labeled probes (*red*) with the desired full length of cDNA populations for both probes (**b**); and undesirable short fragments or unmatched length of labeled cDNA for Cy3 (**c**) or Cy5 (**d**). Sufficient labeling intensities for these reactions as measured by spectrophotometer do not reflect the labeled cDNA population length as examined by the slide gel electrophoresis (This figure is reprinted from Liu and Slininger (2007) page 490, with permission from Elsevier)

dye-labeling concentrations measured by spectrophotometry, but lack of a full length of cDNA populations (Fig. 3c, d) should not be used for hybridization. Stronger signals from one channel will hide the missing signals from the other one. The ratio of signal intensities obtained from the unmatched probe hybridization is misleading and invalid. An equal amount of Cy3- and Cy5-labeling reaction as measured by the Nano-Drop Spectrophotometer should be applied for hybridization. A minimum of 30 pmole each of Cy3- and Cy-5 labeled probes should give sufficient signal intensities under normal conditions.

3.4 Data Acquisition and Normalization

As mentioned above, the reference mini-array is first scanned, and values of the laser PMT gain are balanced for both Cy3 and Cy5 channels before the target scan. If the mini-array is repeatedly exposed to the laser scanner, a slight adjustment will be needed, which accounts for the reduced signal intensities on the mini-array due to the dye bleaching by over exposure to the laser. After a full scan of the target array, each spot on the target array should be examined individually and adjusted or flagged as necessary. Such acquired raw data needs to be normalized using the reference gene *CAB* (CtrlGm_5) embedded in the target array for each gene. Mean signal intensities of *CAB* should be used. Median of foreground signal intensity subtracted by background for each dye channel is applied. Data then should be

filtered between each dye channel and among multiple microarray experiments using a threshold acceptable at a minimum signal intensity level for both channels, for example, a minimum signal intensity of 50 pixels. A gene list shared by all microarray experiments should be generated and used for data analysis. The number of genes on the list is usually smaller than that of the whole genome depending upon the overall quality and variations of the microarray experiments. A gene list representing at least 80% of the genome is reasonable for a quality analysis given the multi-source variations of the assay. The expression patterns of the control genes embedded in the target array relative to the entire genome should be consistent with that of the mini-array (Fig. 2e). As one can easily visualize that without such a reference system, it is impossible to distinguish legitimate differential expressions, especially under stress conditions.

4 Real-Time qRT-PCR Assay

Methods described in this section are optimized using Applied Biosystems 7,500 Real-Time PCR Systems. Variations of performance may exist on other systems and necessary adjustifications and validation may be needed.

4.1 Calibration of Linear Dynamic Range

In order to cross-examine data obtained from microarray and qRT-PCR experiments, primers for qRT-PCR assay-control genes are designed to have the Amplicon/probe overlapped with the 70-mer oligos used for microarray assay. These primers are gene specific and can be applied directly for different platforms of qRT-PCR, including SYBR Green I and TaqMan probe-based chemistries (Table 1). Among the six control genes used for microarray, five genes, *ACTB, B2M, CAB, MSG*, and *RBS1* are selected for qRT-PCR application based on their robust performance and gene specificity. The mRNA can be in vitro transcribed for each of the five reference genes using additional primers and procedures (Liu and Slininger 2007). Due to the higher sensitivity of qRT-PCR assays, a detection range for lower mRNA abundance from 0.1 pg (100 fg) to 1,000 pg is used. For convenient use, a control mix can be prepared consisting of accurately calibrated mRNA transcripts at 100 fg, 1 pg, 10 pg, and 1 ng per µl for *MSG, CAB, RBS1*, and *ACTB*, respectively. This mix serves as a calibration standard over the linear dynamic range. In this set of control genes, *B2M* is used as a negative control. When running the PCR, adding a pair of *B2M* primers without a *B2M* cDNA template in a genome background should give no amplicons. The functional performance of the control set for qRT-PCR is consistent and independent of experimental conditions (Fig. 4 insert).

A detailed bench top protocol incorporating the reference mRNA into the reverse transcription reaction is available (Liu and Slininger 2007). Briefly,

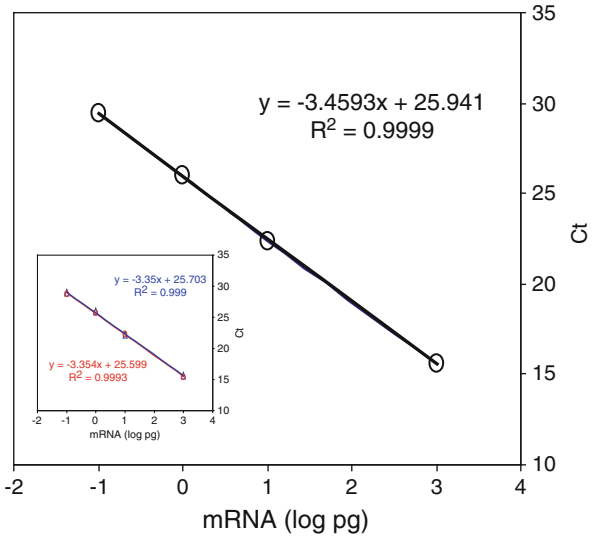

Fig. 4 Functional performance of universal RNA controls for real-time qRT-PCR assays. The inset shows standard curves constructed using robust calibration control genes of *MSG*, *CAB*, *RBS1*, and *ACTB* at 0.1, 1, 10, and 1,000 pg with (○) and without (Δ) 5-hydroxymethylfurfrual challenges in *Saccharomyces cerevisiae* RNA background showing consistent performance of the control genes independent from the toxic challenges. The main panel shows an example of a master equation of standard curves obtained using over 80 individual 96-well plate reactions with and without ethanol stress challenges for yeast demonstrating highly fitted linear relationship between the mRNA input (log pg) of the robust external RNA controls and the PCR cycle threshold (Ct) on ABI 7,500 real time PCR System

the reverse transcription reaction is prepared by adding 1 μl of an accurately prepared mRNA transcript mix into 2 μg of a host total RNA, 0.75 μg of oligo $(dT)_{18}$, and 10 mM of dNTP mix. In reactions for a bacterial background, a 1.5 μg of random primers is used in the place of the oligo $(dT)_{18}$. Adjust the volume by water to 13 μl, then mix well and incubate at 65°C for 5 min. The reaction tubes are chilled on ice for at least 1 min and the following reagents added: 4 μl 5X first strand buffer, 1 μl 0.1 M DTT, 1 μl SuperScript III (200 U/μl) (Invitrogen, CA), and 1 μl RNaseOUT (40 μ/μl) (Promega, WI). The final volume of the reaction is 20 μl. The volume of this reverse transcription reaction can be proportionally increased to at least 80 μl as needed for consistent performance. The reaction is incubated at 50°C for 1 h, 70°C for 15 min, and 4°C to end the reaction using a PCR thermocycler. PCR inhibition related with reverse transcriptase has been observed (Suslov and Steindler 2005), especially for small amounts of RNA or rarely expressed transcripts. Care should be taken, such as using an additional purification step of the reaction product cDNA, particularly when an abnormal PCR amplification efficiency is observed. A standard PCR profile should be sufficient to obtain satisfactory amplification outcomes.

4.2 Robust PCR CT Reference

When different experimental data are generated using qRT-PCR, variations of multiple plate reactions complicate interpretation of data. It is impossible to compare data from different data sets. The key problem is the need of a standard reference for data normalization. For conventional practice of qRT-PCR data acquisition, the PCR cycle threshold (CT) is rather arbitrarily setup by users. It depends upon discretion of individual researchers, and thus data obtained are often not reproducible, especially for quantitative analysis. For example, there are two CT setting options available using the ABI 7,500 PCR System, the *Auto* and the *Manual* options. Under the *Auto* setting option, a default value is taken based on overall reaction performance on a 96-well plate. Such a value varies each time based upon composition and performance of the tested target genes on the same plate. For the *Manual* setting option, users will have to set up a threshold baseline targeting the approximate midpoint of the linear phase of overall reactions. When a user has difficulty choosing between the two methods, the *Auto* option is often used. Unfortunately, the *Auto* is not a good choice. Under the current *Auto* option, it is difficult to repeat the same CT baseline for multiple runs or by multiple users.

The recent development of the robust mRNA standard provides an alternative solution (Liu et al. 2009a). The mRNA reference *CAB* is a unique gene that has no sequence similarities with available microbial genomes. Since it performs consistently in different host genome backgrounds, *CAB* can be designated as a sole CT baseline reference for qRT-PCR data acquisition. The above described calibration control mix containing 1 pg of *CAB* can be used with no additional reagents required. At the data acquisition step, the *Manual* option, but not the *Auto* option provided by the manufacture's built-in program, should be applied. The user can simply bring the PCR cycle threshold baseline to meet the *CAB* reaction curve at a crossing point of cycle number 26 (Fig. 5). This crossing point should always be in the middle of the linear phase of the *CAB* reaction curve. At this point, a normalization analysis can be applied to the reactions on an entire plate. The known amount of *CAB* transcript shows a constant amplification profile that can be applied to any set of plate reactions as a reliable quantification reference. Thus, data obtained from multiple sources or different reaction sets can be unified for comparative analyses. As exemplified using yeast genes, significantly greater variations were observed by using the *Auto* option compared with those by using the *Manual* option (Liu et al. 2009a) (Table 2). Therefore, the built-in *Auto* option for data acquisition and normalization is not recommended for quantitative analysis using qRT-PCR.

4.3 The Master Equation of Standard Curves

For absolute mRNA quantitative analysis, a standard curve is required. Construction of such a standard curve is necessary for each experimental condition using the

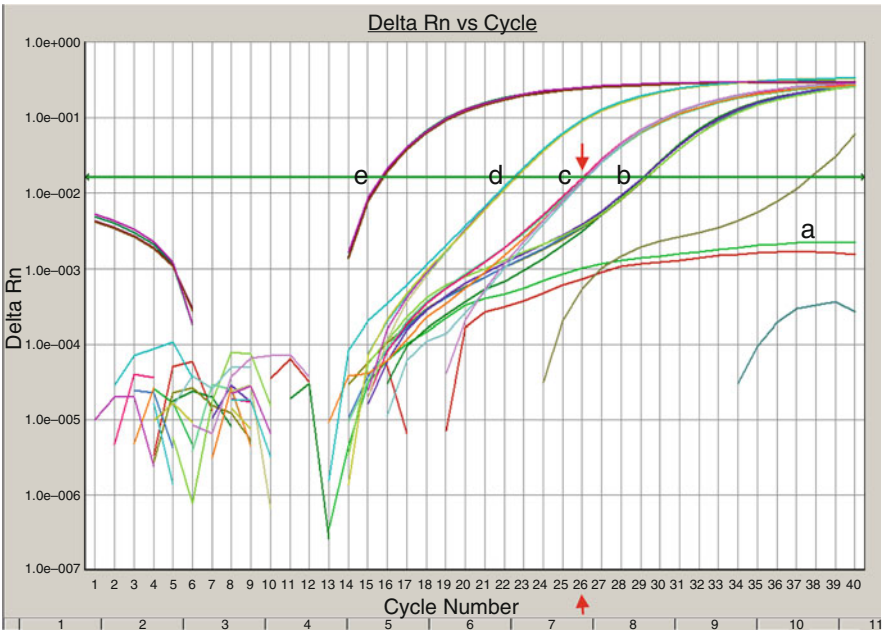

Fig. 5 A typical amplification plot of five control genes on an ABI qRT-PCR 7,500 system showing performance of a four replicated non-template negative control *B2M* (**a**), and five replicated each of *MSG*, *CAB*, *RBS1*, and *ACTB* at 0.1 (**b**), 1 (**c**), 10 (**d**), and 1,000 pg (**e**), respectively. The sole reference for PCR cycle threshold, *CAB* (**c**), was designated to serve as a manual threshold at 26 Ct (indicated by arrows) for a constant data acquisition and analysis for each qRT-PCR run (This figure is reprinted from Liu et al. (2009a) page 13, with permission from Elsevier)

Table 2 Comparison of PCR cycle threshold (CT) variations applying the Auto setting option and the Manual setting option using the robust mRNA *CAB* as the sole CT baseline setting reference for data acquisition in multiple runs of qRT-PCR for *Saccharomyces cerevisiae*

Gene	Normal control				Toxic inhibitor stress			
	Manual		Auto		Manual		Auto	
	Ct	stdev	Ct	stdev	Ct	stdev	Ct	stdev
Control Gene								
MSG	28.98	0.264	30.33	1.197	28.87	0.356	30.40	1.217
CAB	26.06	0.502	27.29	1.218	25.89	0.358	27.19	1.142
RBS1	22.31	0.206	23.47	1.404	22.40	0.306	23.64	1.036
ACTB	15.69	0.184	16.34	1.090	15.51	0.270	16.08	1.064
Target Gene								
CHA1	23.99	0.429	25.90	1.559	19.69	0.408	21.65	1.800
SNQ2	19.04	0.235	20.33	1.360	15.63	0.158	16.38	1.325
PDR3	21.38	0.419	22.83	1.592	16.69	0.186	17.59	1.120
PDR5	20.28	0.537	21.40	1.234	19.97	0.322	21.42	1.638
ADH7	22.49	0.540	23.97	1.757	17.08	0.242	18.45	1.735
ARI1	19.34	0.386	20.51	1.291	17.61	0.324	18.72	1.354
GPM1	15.47	0.119	16.10	1.104	15.99	0.178	16.83	1.292
RPN5	18.59	0.226	19.82	1.488	17.47	0.292	18.79	1.638

qRT-PCR method (Collins et al. 1995; Applied Biosystems 2004). The standard curve is believed to be reliable for the qRT-PCR data processing (Larinov et al. 2005). A large number of reaction wells are needed for standard curve construction therefore reducing available wells significantly for target gene tests. The application of the CT baseline reference, *CAB*, and the universal RNA controls allowed further development of a master equation to overcome this burden (Liu et al. 2009a). A master equation of standard curves can be obtained using the total sum of data acquired by the CT reference. An example of a master equation for *S. cerevisiae* applications is presented as follows:

$$Y = 25.67 - 3.3508X(R^2 = 0.9982) \quad (1)$$

where X represents log mRNA (pg), and Y equals the CT baseline of the qRT-PCR. The master equation is accurate and highly fitted for a linear relationship (Fig. 4). It is independent from several stress conditions including toxic chemical inhibitors. The system performance is robust as measured by the slope and intercept of standard curves over multiple reactions. The efficiency of PCR amplification is sufficiently within the assay capacity (Applied Biosystem 2006, Liu et al. 2009a). Therefore, data of multiple sources can be unified for comparable analyses. This makes a pathway-based qRT-PCR array assay possible (see a later section).

4.4 PCR Amplification Efficiency

PCR amplification efficiency is often neglected when using qRT-PCR assays. However, it impacts quantitative mRNA estimate significantly, particularly when unifying multiple sets of data. Increased concern for the qRT-PCR performance has resulted in the development of numerous algorithms focused on PCR amplification efficiency (Livak and Schmittgen 2001; Pfaffl 2001; Liu and Saint 2002; Tichopad et al. 2003). In fact, PCR amplification efficiencies vary significantly among genes within the same genome. Calculation of amplification efficiency for each gene aids estimation of data quality but does not resolve assay performance problems. Nonetheless, adequate amplification efficiency for the reference genes is necessary to normalize overall reactions in a RNA background. For qRT-PCR, a 10% variation of amplification efficiency is acceptable for the assay (Applied Biosystems 2006). The amplification efficiency can be monitored by the slope of the master equation efficiently or calculated by users using a conventional standard curve (Table 3). The valid threshold for slope ranges between −3.58 and −3.12 reflecting amplification efficiency from 90 to 110%. And the optimum slope for amplification efficiency should fall between −3.33 and −3.32. Accordingly, if the slope shows a value beyond the defined threshold for a set of data, the data are unreliable and cannot be applied for a comparative analysis.

Table 3 PCR amplification efficiency as indicated by the slope of linear regression relationships

Slope	Exponent amplification	Amplification efficiency[a]
−3.58	1.9025	0.9025
−3.53	1.9199	0.9199
−3.48	1.9380	0.9380
−3.43	1.9568	0.9568
−3.38	1.9763	0.9763
−3.33	1.9966	0.9966
−3.32	2.0008	1.0008
−3.27	2.0221	1.0221
−3.22	2.0444	1.0444
−3.17	2.0676	1.0676
−3.12	2.0918	1.0918
−3.11	2.0967	1.0967

[a]Amplification efficiency (E) was calculated using equation $E = [10^{\wedge}(-1/\text{slope})] - 1$

5 Unification of Gene Expression Data for Comparable Analyses

The ultimate goal of applying mRNA reference is to unify and compare data obtained from different reaction sources for analyses. Such unification implies data obtained within each platform such as microarray or qRT-PCR individually as discussed below. On more advanced applications, it is also possible to combine and compare expression data across different plateforms of microarray and qRT-PCR.

5.1 Microarray

Unification of gene expression data is mainly used for assembling data obtained from different experimental conditions and sets within a specific high throughput assay platform such as microarray or qRT-PCR for comprehensive analyses. Microarray assay is suitable for transcriptome level investigations with relatively high mRNA abundance. A lower detection limit is defined at 10 pg level for microarray analysis (Choi and Tiedje 2002; Liu and Slininger 2007). The linear range of signal intensities detected by microarray is validated between 10 and 7,000 pg. As described previously, after data normalization, a data filtration is necessary to generate a shared gene list. The filtered gene list shared by both channels for multiple microarray experiments is the key for data comparability. Computational algorithm for data normalization and analysis is beyond the scope of this chapter. However, based on the normalization procedures using the reference genes, data are reproducible and can be unified for comparative analysis. The unified data then can be analyzed using a suitable program, such as commercial software package, on-line free software, or special designed programs, for in-depth analyses.

5.2 Real-Time qRT-PCR and PCR Array

Compared with microarray assay, the detection limit of qRT-PCR assay is approximately 100 times more sensitive than that of the microarray. Its valid detection ranges from 100 fg to 1,000 pg (Liu and Slininger 2007). Applying the universal RNA control and the robust data acquisition reference, once the reaction completed, data can be exported to an Excel file and treated using a customized macro Visual Basic function for basic statistical analyses (Liu et al. 2009a). Data can be pooled from multiple sources as described (Liu et al. 2009b; Ma and Liu 2010). Comparison of mRNA expression is commonly presented by relative fold changes. Using the robust CT acquisition reference and the master equation, expression of a gene can be estimated in absolute mRNA mass in pg. Alternatively, the mRNA mass can be readily converted to a gene transcript copy number using a modified equation (Staroscik 2004; Liu et al. 2009a) as follows:

$$\text{Gene copy number} = [\text{mRNA(pg)} * 6.022 \times 10^{20}]/[\text{Amplicon(bp)} * 1 \times 10^{9} * 650] \quad (2)$$

where mRNA is an estimated numeric value in pg using the master equation and Amplicon is the bp-length of an amplified target gene. An executable computer program using C++, MasterqRT-PCR, is developed and freely available to perform comprehensive tasks of the control system as described (Liu et al. 2009a). Materials of the universal RNA controls, including the robust data acquisition reference and bench top protocols, are available at NCAUR USDA-ARS with no monetary charges to qualified queries.

Thanks to the robust CT baseline reference and the master equation of standard curves, it is possible to develop a pathway-based qRT-PCR array assay for evaluation of a large number of subset genes under different experimental conditions. The consistent performance of PCR using SYBR Green I suggests the method can be a popular choice for a large number of single-gene assays due to its cost savings over that for the additional fluorescent probes required by TaqMan probe method. It needs to be pointed out that gene-specific primers and optimized reaction conditions are critical when the SYBR Green method is used. Examples of PCR array assays and detailed application descriptions are available elsewhere (Liu et al. 2009b; Ma and Liu 2010).

5.3 Comparison Across Different Assay Platforms

Relatively few systematic studies comparing expression data across different platforms are available. When comparison of expression data from microarray and qRT-PCR is made, results are usually not consistent (Etienne et al. 2004; Bammler et al. 2005; Dallas et al. 2005; Irizarry et al. 2005; Larkin et al. 2005).

One major cause is that each assay method uses different reference controls for data handling. In addition to the assay variations, when inconsistent control gene sequences are used for normalization for different assay platforms, the derived results are likely inconsistent and not comparable. Sequence specificity affects efficiency of nucleotide hybridization. If a PCR amplicon does not overlap with the microarray probe sequence, data obtained from qRT-PCR and microarray are not well correlated (Etienne et al. 2004). When overlapping sequences are used for the same gene, data from the two platforms show better agreement (Etienne et al. 2004; Dallas et al. 2005; Liu and Slininger 2007). Therefore, the same DNA sequence or at least overlapping sequences that can be measured by microarray and qRT-PCR assays, as described earlier, should be used as reference controls for gene expression comparison analysis across different platforms.

Another important factor affecting the comparability of the data is the sensitivity of each assay platform. As mentioned above, a microarray has valid detection dynamics for higher mRNA levels in the range from 10 to 7,000 pg, whereas qRT-PCR has a better detection range at lower levels from 100 fg to 1,000 pg. The qRT-PCR method is 100 times more sensitive than microarray for the lower detection limit. On the other hand, the capacity of microarray assay is seven times greater at the higher abundance levels. A poor correlation of the expression data across microarray and qRT-PCR may not be surprising if the comparison is beyond the capacity of the assay (Czechowski et al. 2004; Etienne et al. 2004). The overlapping detection ranges shared by the two assays are between 10 and 1,000 pg for both qRT-PCR and microarray methods. Therefore, a valid comparison of quantitative gene expression using the same RNA sample is possible for the two methods, only within this overlapping linear range of detection. Consistent correlation of expression data by microarray and qRT-PCR was observed in yeast under the inhibitor stress (Liu and Slininger 2007).

5.4 Troubleshooting

For each assay platform, a validation of expression procedure is necessary since instrument designs vary and program compatibilities differ as well. For example, certain qRT-PCR systems show lower amplification efficiencies and adaptation and adjustment are required. As tested by an ABI system, for core facility and home-made microarray use, this control system is readily applicable. For commercial microarray assay service, the control system needs to be incorporated into the manufacturer's template. For example, Microarrays Inc. (Huntsville, AL) recently adapted the reference format for customized yeast microarray fabrications (personal communications). In contrast to a huge amount of technical descriptions available for microarray and qRT-PCR assays, problems related to quality control and reference genes are rarely addressed. For user's convenience, a brief guideline of troubleshooting on the reference gene and quality control related issues is presented (Table 4).

Table 4 A quick reference guide of troubleshooting for common problems related to quality control issues in gene expression analysis of microbial stress tolerance

Problem	Possible cause	Solution
Microarray		
Data do not make sense	Impropriate cell collection	Collect cells at experimental temperature and freeze cell samples immediately on dry ice. Do not centrifuge cells at 4°C for cell collection
Lack of long cDNA species	RNA degradation	Isolate and purify high quality RNA
Low signal intensities	Insufficient labeled probes for hybridization	Use a minimum of 30 pmole equal amount of Cy3 and Cy5 labeled probes for hybridization
Cy3 or Cy5 signal too strong	Imbalanced input of labeled probes	Quantify equal amount of Cy dye labeled probes for hybridization
Data not reproducible	Unmatched probe labeling	Use sGel to evaluate every probe used for hybridization
Signal intensities fade after final scanning	Imbalanced dye bleach especially for Cy5	Use mini-array test before a full scan of the target array
Inconsistent data output	Impropriate normalization or data filtration	Use mean value of *CAB* to normalize every gene and a common accepted minimum signal intensity to filter both dye channels
Data inconsistent with database	Database out of date or impropriate extraction algorithm	Refer to the original research report for fact
qRT-PCR		
Random variations on a plate	Malfunction of qRT-PCR machine	Calibrate the qRT-PCR machine periodically
Control Ct too low or too high	Variation of mRNA input	Calibrate and use a set of designated accurate levels of pipettes
Reactions not reproducible	Batch variation/aging of reagents	Use the same batch and fresh reagents
High levels of variation	Variations of RT reactions	Use the same reverse transcription reaction as template. The RT volume can be proportionally increased up to 80 μl per reaction as desired
Variations of technical replications	Operation error	Make master tube mixtures and aliquot carefully
MasterqRT-PCR does not function	Incompatible reaction template	Use suggested template format

6 Conclusion and Perspectives

Over the past decade, significant efforts and development on external RNA control reference provide useful tools of quality control system for enhanced gene expression technology, including the universal RNA controls, the robust *CAB* qRT-PCR CT reference, the master equation, and associated analytical methods and programs. The performance of these quality controls is consistent and independent from different experimental conditions and environmental stimuli. It simplifies the assay procedures, and safeguards data fidelity and reproducibility allowing unification of expression data from multiple experimental sources for comparable and

comprehensive analyses. As a result, it is possible to develop pathway-based qRT-PCR array assays. The control system can be applied to different platforms of microarray and qRT-PCR, including SYBR Green I and TaqMan probe-based chemistries. The mRNA mass-detection limit is defined from 10 to 7,000 pg and 100 fg to 1,000 pg for microarray and qRT-PCR, respectively. Comparisons of data between the two platforms can be made within the valid overlapping detection range from 10 to 1,000 pg. The mRNA mass estimate can be readily converted to gene transcript copy numbers as desired. Rapid advances of genomics and computation biotechnology have fundamentally changed the way scientists address biological questions today. The exponentially growing volume of expression database is an ideal subject for comprehensive analyses using integrated tools, such as bioinformatics (Kapushesky et al. 2010) and systems biology, to draw fundamental principles of life events. Unification of gene expression data using a quantification reference is necessary; and every piece of expression data block generated by independent research is accountable toward building the invaluable expression databases for the community.

Acknowledgment The author is grateful to Michael A. Cotta and Marsha Ebener for proofreading of the manuscript. This work was supported in part by NIFA National Research Initiative Award 2006-35504-17359. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Applied Biosystems (2004) Absolute quantification: Getting started guide for the 7300/7500 System, Part Number 4347825 Revison A. Foster City

Applied Biosystems (2006) Amplification efficiency of Taqman gene expression assays, Application Note 5 pp Publication 127AP05-03. Foster City

Baeber RD, Harmer DW, Coleman RA, Clark BJ (2005) GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics 21:389–395

Bammler T, Beyer RP, Bhattacharya S, Boorman GA, Boyles A, Bradford BU, Bumgarner RE, Bushel PR, Chaturvedi K, Choi D, Cunningham ML, Deng S, Dressman HK, Fannin RD, Farin FM, Freedman JH, Fry RC, Harper A, Humble MC, Hurban P, Kavanagh TJ, Kaufmann WK, Kerr KF, Jing L, Lapidus JA, Lasarev MR, Li J, Li Y-J, Lobenhofer EK, Lu X, Malek RL, Milton S, Nagalla SR, O'Malley JP, Palmer VS, Pattee P, Paules RS, Perou CM, Phillips K, Qin L-X, Qiu Y, Quigley SD, Rodland M, Rusyn I, Samson LD, Schwartz DA, Shi Y, Shin J-L, Sieber SO, Slifer S, Speer MC, Spencer PS, Sproles DI, Swenberg JA, Suk WA, Sullivan RC, Tian R, Tennant RW, Todd SA, Tucker CJ, Van Houten B, Weis BK, Xuan S, Zarbl H (2005) Standardizing global gene expression analysis between laboratories and across platforms. Nat Methods 2:351–356

Bower NI, Moser RJ, Hill JR, Lehnert SA (2007) Universal reference method for real-time PCR gene expression analysis of preimplantation embryos. Biotechniques 42:199–206

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R,

Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAME)–toward standards for microarray data. Nat Genet 29:365–371

Bustin SA, Benes VJ, Garson A, Hellemants J, HuggettJ KM, Mueller R, Nolan TM, Pfaffl W, Shipley GL, Vandesompele JC, Wittwer T (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:4

Choi J-C, Tiedje JM (2002) Quantitative detection of microbial genes by using DNA microarrays. Appl Environ Microbiol 68:1425–1430

Collins ML, Zayati C, DetmerJJ DB, Kolberg JA, Cha T-A, Irvine BD, Tucker J, Urdea MS (1995) Preperation and characterization of RNA standard for use in quantitative branched DNA hybridization assays. Anal Biochem 226:120–129

Collins C, Rommens JM, Kowbel D, Godfrey T, Tabnner M, Hwang SI, Polikoff D, Nonet G, Cochran J, Maymbo K, Jay KE, Frooula J, Cloutier T, Kuo WL, Yaswen P, Dairkee S, Giovanola J, Hutchinson GB, Isola J, Kallioniemi OP, Palazzolo M, Martin C, Erricson C, Pinkel D, Albertson D, Li WB, Gray JW (1998) Positional cloning of *ZNF217* and *NABC1*: genes amplified at 20q13.2 and over expressed in breast carcinoma. Proc Natl Acad Sci USA 95:8703–8708

Cronin M, Ghosh K, Sistare F, Quackenbush J, Vilker V, O'Connell C (2004) Universal RNA reference materials for gene expression. Clin Chem 50:1464–1471

Czechowski T, Bri RP, Stitt M, Scheible W, Udvardi MK (2004) Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root-and shoot-specific genes. Plant J 38:366–379

Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM, Cummings AJ, Kees UR (2005) Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate? BMC Genomics 6:59

Ellefsen S, Stenslokken K-O, Sandvik GK, Kristensen TA, Nilsson GE (2008) Improved normalization of real-time reverse transcriptase polymerase chain reaction data using an external RNA control. Anal Biochem 376:83–93

ERCC (2005a) The external RNA controls consortium: a progress report. Nat Methods 2:731–734

ERCC (2005b) Proposed methods for testing and selecting ERCC external RNA controls. BMC Genomics 6:150

Etienne W, Meyer MH, Peppers J, Meyer RA Jr (2004) Comparison of mRNA gene expression by RT-PCR and DNA microarray. Biotechniques 36:618–626

Fisk DG, Ball CA, Dolinski K, Engel SR, Hong EL, Issel-Tarver L, Schwartz K, Sethuraman A, Botstein D, Cherry JM (2006) *Saccharomyces cerevisiae* S288C genome annotation: a working hypothesis. Yeast 23:857–865

Goldsworthy SM, Goldsworthy TL, Sprankle CS, Butterworth BE (1993) Variation in expression of genes used for normalization of Northern blots after induction of cell proliferation. Cell Prolif 26:511–518

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalization: strategies and considerations. Genes Immun 6:279–284

Irizarry RA, Warren D, Spencer F, Kim IF, Biswal S, Frank BC, Gabrielson EJ, Garcia GN, Geoghegan J, Germino G, Griffin C, Hilmer SC, Hoffman E, Jedlicka AE, Kawasaki Martínez-Murillo EF, Morsberger L, Lee H, Petersen D, Quackenbush J, Scott A, Wilson M, Yang Y, Qing Ye S, Yu W (2005) Multiple-laboratory comparison of microarray platforms. Nat Methods 2:345–350

Kakuhata R, Watanabe M, Yamamoto T, Akamine R, Yamazaki N, Kataoka S, Fukuoka S, Ishikawa M, Ooie T, Baba Y, Hori T, Shinohara Y (2007) Possible utilization of in vitro synthesized mRNA s specifically expressed in certain tissues as standard for quantitative evaluation of the results of microarray analysis. J Biochem Biophys Methods 70:755–760

Kanno J, Aisaki K, Igarashi K, Nakatsu N, Ono A, Kodma Y, Nagao T (2006) "Per cell" normalization method for mRNA measurement by quantitative PCR and microarrays. BMC Genomics 7:64

Kapushesky M, Emam I, Holloway E, Kurnosov P, Zorin A, Malone J, Rustici G, Williams E, Parkinson H, Brazma A (2010) Gene expression atlas at the European Bioinformatics Institute. Nucleic Acids Res 38:D690–D698, Database issue

Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8:257–260

Lage JM, Hamann S, Gribanov O, Leamon JH, Pejovic T, Lizardi PM (2002) Microgel assessment of nucleic acid integrity and labeling quality in microarray experiments. Biotechniques 32:312–314

Larinov A, Krause A, Miller W (2005) A standard curve based method for relative real time PCR data processing. BMC Bioinformatics 6:62

Larkin JE, Frank BC, Gavras H, Sultana R, Quackenbush J (2005) Independence and reproducibility across microarray platforms. Nat Methods 2:337–344

Liu W, Saint DA (2002) A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem 302:52–59

Liu ZL (2010) Unification of gene expression data applying standard mRNA quantification reference for comparable analyses. J Microbial Biochem Technol 12:124–126

Liu ZL, Slininger PJ (2007) Universal external RNA quality controls for mRNA expression analysis using microbial DNA oligo microarray and real time quantitative RT-PCR. J Microbiol Methods 68:486–496

Liu ZL, Palmquist DE, Ma M, Liu J, Alexander NJ (2009a) Application of a master equation for absolute mRNA quantification using qRT-PCR. J Biotechnol 143:10–16

Liu ZL, Ma M, Song M (2009b) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282:233–244

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25:402–408

Ma M, Liu ZL (2010) Quantitative transcription dynamic analysis reveales candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Genomics 10:169

Mohsenzadeh M, Saupe-Thies W, Sterier G, Schroeder T, Francella F, Ruoff P, Rensing L (1998) Temperature adaptation of house keeping and heat shock gene expression in *Neurospora crassa*. Fungal Genet Biol 25:31–43

Novoradovskaya N, Whitfield ML, Basehore L, Novoradovsky SA, Pesich RJ, Usary J, Karaca M, Wong WK, Aprelikova O, Fero M, Perou CM, Botstein D, Braman J (2004) Universal reference RNA as a standard for microarray experiments. BMC Genomics 5:20

PE Applied Biosystems (1997) User Bulletin #2 Norwalk. Perkin-Elmer Corp, CT, p 36

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Smith RD, Brown B, Ikonomi P, Schechter AN (2003) Exogenous reference RNA for normalization of real-time quantitative PCR. Biotechniques 34:88–91

Staroscik A (2004) Availible at http://www.uri.edu/research/gsc/resources/cndna.html

Suslov O, Steindler DA (2005) PCR inhibition by reverse transcriptase leads to an overestimation of amplification efficiency. Nucleic Acids Res 33:e181

Tichopad A, Dilger M, Schward G, Pfaffl MW (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Res 31:e122

Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA, Orlando C (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309:293–300

VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44:619–626

Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. Biotechniques 39:1–11

Index

A
AASM. *See* Amino acid scanning mutagenesis
Acetate, 59, 162, 164–172
Acetic acid, 63, 214
Acetic acid-induced programmed cell death, 62–69
Acetic acid-programmed cell death, 68
Acetone-butanol-ethanol (ABE) fermentations, 184
ACTB, 285
Adaptation to acetic acid, 61
ADP/ATP carrier, 67
AFEX. *See* Ammonia fiber expansion
Aldehyde inhibitors, 1–2
Aldehyde reductase, 3
Alternative PCD pathways, 68
Amino acid scanning mutagenesis (AASM)
  in vitro expression, 264
  Lyt–1 gene, 264, 265
  mutagenized ORFs, 264
  oligonucleotides, 264, 265
  vs. randomized mutagenesis, 264
Ammonia fiber expansion (AFEX), 211
Amplicon, 288
Assay, 295

B
Batch, 120, 126, 129–130
Benzoate, 59
Beta–2-microglobulin (B2M), 285
BioBricks, 227
Biodiesel, 218
Biofuels, 1, 58, 162, 212
Bio-gasoline, 218
Bio-jet fuel, 218
Bioprocess engineering, 118–119, 124, 126–131
Biosensor, 213, 226
B2M. *See* Beta–2-microglobulin
Butanol, 219–220

C
CAB, 285
CALB. *See Candida antarctica* lipase B
C5–10 alcohols and hydrocarbons, 220–221
cAMP, 103–105
Candida antarctica lipase B (CALB), 272–273
Caspase-like activities, 66, 68
Catalase, 64, 65
cDNA, 286
Cell adaptation, 57
Cell cycle, 30, 44, 46, 47
Cellulosic, 162
Cell wall, 79–88
Chaperones, 21, 95–98, 222
Chemical induced chromosome expansion (CIChE), 213, 228
Chromosomal integration, 228
CIChE. *See* Chemical induced chromosome expansion
Cis/*trans* isomerase, 222
Coenzyme specificity, 143, 144
Cofactor, 14, 82, 102–103
Compatible solute, 121
Consecutive batch fermentation, 129–130
Continuous fermentation, 120, 123–124, 131
CT. *See* Cycle threshold
Cy3, 286
Cy5, 286
Cycle threshold (CT), 290

Z.L. Liu (ed.), *Microbial Stress Tolerance for Biofuels*, Microbiology Monographs 22,
DOI 10.1007/978-3-642-21467-7,

Cyt c. *See* Cytochrome c
Cyt c release, 66
Cytochrome c (cyt c), 64, 66, 67
Cytochrome c oxidase, 67

D
Data acquisition, 287–288
Death pathways, 67
Desaturase, 221
Dilute acid, 211
Discriminant function analysis (DFA), 253

E
E. coli. *See Escherichia coli*
Efflux, 217
Efflux pumps, 215, 222, 223, 226
End product toxicity, 228
EnvZ, 224
Ergosterol, 79, 84, 97
Escherichia coli (E. coli)
  gram-negative bacterium, 187
  isolation and gene set expression, 183
  mutants, 192
Ethanol, 13–14, 162–165, 168–171, 219
Ethanol inhibition, 118–119, 121, 123–124, 127, 129–131
Evolutionary engineering, 146–147
External RNA control, 281–282

F
FDM. *See* Furan–2,5-dimethanol
Fed-batch, 120, 123–126
FixL, 224
Fluorescent transcriptional reporters, 225
FM. *See* 2-Furanmethanol
Food preservatives, 58
Fps1p aquaglyceroporin channel, 61
Function, 22
Functional
  categories, 22
  group, 22
Furan, 213
Furan–2,5-dimethanol (FDM), 3, 13
2-Furanmethanol (FM), 3, 13
Furfural, 2

G
Gas chromatography coupled with mass spectrometry (GC-MS)
  identification and quantification, metabolites, 244–245
  industrial continuous and batch fermentation
    environmental stress, 246–247
    multivariate statistical analysis, 247
  inoculum densities
    cell-density fermentation, 245
    high inoculum size, 246
    inoculation density optimization, 246
    quenching, 245
  principles
    electrospray ionization (ESI), 243
    spectral libraries, 242
  sample preparation
    quenching methods, 244
    trimethylsilyl (TMS), 244
  vacuum fermentation, 248
Gene, 19–20
Gene expression, 3, 279
General weak acid stress response, 58
Genome, 22, 280
Genomic adaptation, 17
Genomics, 161–172
Genotypes, 279
Global transcription machinery engineering (gTME)
  description, 191
Glucose–6-phosphate dehydrogenase (G6PDH), 145
Glyceraldehyde 3-phosphate dehydrogenase, 145
Glycerol–3-phosphate dehydrogenase, 62
Glycine betain, 221
Glycogen, 29–48
  glycogenin, 31–32, 47
  glycogen phosphorylase, Gph1, 32, 34–36, 42
  glycogen synthase Gsy1, Gsy2, 32–35, 42–43
  UDP-glucose, 38–39
Glycolysis, 14, 85, 97–102
G6PDH. *See* Glucose–6-phosphate dehydrogenase

H
HCA. *See* Hierarchical clustering analysis
Heat shock element (HSE), 106–107
Heat shock proteins (HSPs), 21, 79, 95–98, 125
Hexose transporter, 143, 148
hfq, 168–170, 172
HG. *See* High gravity

Hierarchical clustering analysis (HCA), 252
High gravity (HG), 118–132
High-osmolarity-glycerol (HOG) pathway, 121–123
*him*A, 170–171
HMF. *See* 5-Hydroxymethyl–2-furfural
Hog1p, 61
HOG pathway. *See* High-osmolarity-glycerol pathway
HSE. *See* Heat shock element
HSPs. *See* Heat shock proteins
Hydrogenperoxide (H_2O_2), 64
5-Hydroxymethyl–2-furfural (HMF), 2

I
Immobilized cells, 128–129
Impairment in mitochondrial functions, 67
Industrial processing stresses, 125–126
Inhibitor, 161–172
Inhibitory inhibitors, 119, 120, 128
In situ detoxification, 1–2
Intracellular acidification, 59
Ionic liquid, 211, 213

K
Kinetics, 124

L
LC-MS. *See* Liquid chromatography coupled to mass spectrometry
Lignocellulosic biomass, 118, 119, 127, 128, 131–132
Linear dynamic ranges, 282
Lipidomics
  LC-MS, 250
  membrane lipids, 250
  yeast, inhibitors
    fluidity regulation, 251
    membrane lipids changes, 250
    phosphatidylinositol (PI), 251
    wavelet-principle components analysis (WT-PCA), 251
Lipophilic carboxylate compounds, 61
Liquid chromatography coupled to mass spectrometry (LC-MS)
  analysis
    atmospheric pressure chemical ionization (APCI), 249
    mass signals, 249
  lipidomics development, 249
Lycotoxin–1 (Lyt–1)
  AASM, 264, 265
  CALB, 272–273
Lyt–1. *See* Lycotoxin–1

M
MAGE. *See* Multiplex automated genomic engineering
Master equation, 290–292
Medium optimization, 127–128
Membrane, 78–88, 94, 97–99
  structures, 124
Metabolic engineering, 163, 171, 172
Metabolism, 14
Metabolomics, ethanologenic yeast
  bibliometric analysis, 241
  data mining
    supervised, 253
    unsupervised, 252
  GC-MS, research strategy
    identification and quantification, metabolites, 244–245
    industrial continuous and batch fermentation, 246–247
    metabolomic analysis, inoculum densities, 245–246
    principles, 242–243
    sample preparation, 244
    vacuum fermentation, 248
  genomics, transcriptomics, and proteomics, 240
  LC-MS, research strategy
    fundamentals, 249
    lipidomics, 250
    lipidomic study, inhibitors, 250–251
  methods and procedures, 242, 243
  NMR, 252
  potential biomarkers, 253
  stress tolerance and strain productivity, 254
Metacaspase-encoding YCA1 gene, 65–66
Metagenomic fosmids, 229
Microarray, 165, 166, 280
Microbial solvent tolerance
  biofuels applications
    "biofuel substrates" and potential products, 196–197
    butanol fermentation bioprocesses, 198
  biological application and relevance
    butanol, 182–183
    cellulose and hemicellulose, 182
    Clostridium spp., 183
    first and second generation ethanol, 182
    phenolic compounds, 184

Microbial solvent tolerance (*cont.*)
bioremediation, 178
communities and consortia
ecosystem, 195
hydrocarbon biodegradation, 196
PAH, 196
ethanol production, 181
exclusion
AcrB, 187–188
E. coli, 187
membrane fusion protein/AcrA, 188
prokaryote species, 188
RND-type efflux pumps, 187
"general cell response", 199
isolation/cultivation approaches, 190
log*P* value, 185
membrane adaptation
cis–trans isomerization, unsaturated fatty acids, 185–186
yeast cells, 186
mesophilic bacteria
E. coli, 192
genus *Lactobacillus*, 192
Pseudomonas species, 191–192
microalgae and cyanobacteria, 194–195
"normal" prokaryotes, 181
production, commercial/research value strains, 178–180
regulation, response
cis–trans isomerase activity, 189
efflux pump proteins expression, 189
saturated and existing membrane fatty acids, 198
thermophilic bacteria
C. thermocellum, 193–194
genus *Geobacillus*, 194
Thermoanaerobacter ethanolicus mutant strain, 194
yeasts
improved ethanol, 190–191
STRE and gTME, 191
Mini-array, 283
Mitochondria, 66–69
Mitochondrion, 79, 80, 82, 87–88, 93, 98
Mitogen-activated protein (MAP) kinases, 61
mRNA, 279
MSG, 285
Multiplex automated genomic engineering (MAGE), 228

N

N-acetyl-L-cysteine, 65
NADH. *See* Nicotinamide adenine dinucleotide
NADPH. *See* Nicotinamide adenine dinucleotide phosphate
Necrosis, 63
*nha*A, 164–170, 172
Nicotinamide adenine dinucleotide (NADH), 12, 79, 101, 102
Nicotinamide adenine dinucleotide phosphate (NADPH), 12, 101, 102
Normalization, 280
Nuclear transcription factor War1p, 61

O

Octanol–water partition coefficient, 218
Oleic acid, 79, 84
Open reading frames (ORFs), *S. cerevisiae*
anaerobic growth, xylose, 269–270
full-genome collection, 269, 270
robust microbial strains, 268
XI expression, 269
ORFs. *See* Open reading frames
Orthogonal signal correction (OSC), 253
Oscillations, 43–44
Osmotic stress, 120–123, 130–132

P

PAH. *See* Polyaromatic hydrocarbons
Pathway, 4
PCA. *See* Principal components analysis
PCD. *See* Programmed cell death
PCD in response to acetic acid, 58
PCR. *See* Polymerase chain reaction
Pentose phosphate pathway, 14
Pentose phosphate pathway (PPP), 85, 97, 99, 101–102, 139, 144–147, 151, 152
Peroxisome, 79, 81, 88, 93
6PGDH. *See* 6-phosphogluconate dehydrogenase
PGK1. *See* Phosphoglycerate kinase 1
Phenolic, 214
Phenotypes, 279
6-phosphogluconate dehydrogenase (6PGDH), 145
Phosphoglycerate kinase 1 (PGK1), 285
PKA. *See* Protein kinase A
Plasma membrane transporter Pdr12p, 61
Plasmid-based functional proteomics, biofuel production
description, 260
recombinant *S. cerevisiae*
lignocellulosic feedstocks, 266
lipase B production, 271–273
production, ORFs, 268–270

protein binding properties, 271
SUMO, 266–268
robotic workcell
AASM, 264–265
automated molecular biology protocols, 263–264
plasmid preparation, 261–263
Platforms, 295
Polyaromatic hydrocarbons (PAH)
environmental contaminants, 178
Polymerase chain reaction (PCR)
amplification, 292
amplification efficiency, 292
array, 294
PPP. *See* Pentose phosphate pathway
Pretreatment, 161–172
Principal components analysis (PCA)
pattern recognition method, 253
phases, 247
regression extension, 253
unsupervised techniques, 252
Probes, 286
Pro-death factors, 66
Programmed cell death (PCD), 62–69
Proline, 88, 94–95, 97
Proteasome, 66
Protein kinase
Pho85, 30, 33, 34
PKA, 30, 33, 37, 39–40
Psk1,2, 33
Snf1, 30, 39, 41
TOR(C1), 30, 39–42
Protein kinase A (PKA), 84, 103–105
Proton-translocating ATPase Pma1p, 59
Pyrophosphates, 217

Q

qRT-PCR. *See* Quantitative reverse transcription polymerase chain reaction
Quality control, 283
Quantitative reverse transcription polymerase chain reaction (qRT-PCR), 280

R

Reactive oxygen species (ROS), 64–65
Recombinant *Saccharomyces cerevisiae*
lignocellulosic feedstocks, 266
lipase B production
biodiesel, 271–272
CALB gene ORF expression, 272, 273
oligonucleotide PCR assembly strategy, 272, 273
one-step-charging resin, 272–273
transesterification reaction, 271
production, ORFs
anaerobic growth, xylose, 269–270
full-genome collection, 269, 270
robust microbial strains, 268
XI expression, 269
protein binding properties
hybrid interaction protocol, 271
pentose phosphate pathway, xylose, 271
Piromyces XI, 271
SUMO, three-plasmid yeast expression system
gene optimization and AASM, 267, 268
pSUMOduo/URA, 267, 268
screen engineered strains, 267
XI and XKS expression, 268
xylose and arabinose fermentation, 266
Redox balance, 2
Reference gene, 281, 287
Regulation, 21
Regulatory networks, 23, 280
Reprogrammed pathways, 2
Resistance nodulation cell division (RND)
efflux pumps, 188
RND-type efflux mechanisms, 187
Ribonucleic acid (RNA), 288–289
Ribulose
5-phosphate 3-epimerase (RPE1), 141, 151
5-phosphate ketol-isomerase (RKI1), 141, 151
Ribulose bisphosphate carboxylase small chain 1 precursor (RBS1), 285
RNA. *See* Ribonucleic acid
Robotic workcell
AASM
in vitro expression, 264
Lyt–1 gene, 264, 265
mutagenized ORFs, 264
oligonucleotides, 264, 265
vs. randomized mutagenesis, 264
automated system requirements, 261
high-quality plasmid DNA, 261
integrated, 262
molecular biology protocols
development and screening, 263
Lyt–1 gene, 263–264
plasmid preparation
cellulase F *(celF)* gene, 261–262
cellulase mutants, 261

Robotic workcell (*cont.*)
screening assay, 263
96-well format, 261
ROS. *See* Reactive oxygen species

S
Saccharomyces cerevisiae, 11, 58
Scaffolding, 217
Selected reaction monitoring (SRM), 226
Self-flocculation of yeast cells, 129
sGel, 286
Signal intensities, 286
Simultaneous saccharification and fermentation (SSF), 120, 131, 132
SLIC/Gibson, 213
Slope, 292
Small ubiquitin-like modifier (SUMO)
gene optimization and AASM, 267, 268
screen engineered strains, 267
XI and XKS expression, 268
SOD. *See* Superoxide dismutase
Sodium chloride (NaCl), 221
SRM. *See* Selected reaction monitoring
SSF. *See* Simultaneous saccharification and fermentation
Standard curve, 290
Starch-based feedstocks, 118–126, 131–132
Strain improvement, 171
STRE. *See* Stress response element
Stress, 21, 280
autophagy, 41
MSN2/4, 39–41, 45
STRE, 40, 42
Stress response elements (STRE), 103, 105–108
and Msn role, 191
S. cerevisiae genes promotor sequence, 191
Stress tolerance, 119, 126, 127, 130
Stuck/sluggish fermentations, 118, 123–124, 127
Sugar-based feedstocks, 118–120, 131
SUMO. *See* Small ubiquitin-like modifier
Superoxide dismutase (SOD), 64
Superoxide protonation, 65
Sustained oscillation, 130
SYBR green, 294
Systems biology, 23, 280
Systems biology tools, 163–165

T
Tanks-in-series system, 120, 121, 123–124, 126
Taqman, 294
Taz1, 224
Thermal stress, 124–125
TodS, 224
Tolerant candidate genes, 124
TPL. *See* Tyrosine phenol-lyase
Transaldolase, 151
Transcription factor, 4
Transcriptopmics, 166
Transcripts, 19, 288
Transketolase, 151
Transporters, 19
Trans unsaturated fatty acids, 221
Trehalose, 29–48, 85, 97, 99–100
TPS/TPP complex, 36
TPS1/Tps1, 36–38
Trehalase, Nth1, Ath1, 32, 37–38
Trehalose 6-phosphate, Tre6P, 36, 45–47
Tryptophan, 88–94, 97
Trz1, 224
Two-component systems, 224
Tyrosine phenol-lyase (TPL)
activity, 184
Pseudomonas putida strains, 184

U
UhbP, 224
Universal RNA controls, 281

V
Vacuolar protease Pep4p, 67
Vacuole, 79, 80, 82, 87, 93
Very high gravity (VHG), 118–121, 124, 127–131
VHG. *See* Very high gravity
VirA, 224

W
Wavelet transform (WT), 253
Weak acid, 58
adaptation, 60
stress, 58–62, 59–60
stress response, 57

X
XI. *See* Xylose isomerase
XK. *See* Xylulokinase
X5P. *See* Xylulose 5-phosphate
Xylitol dehydrogenase, 139–145, 152

Xylose
isomerase (XI), 139, 143, 145–147, 150–152
metabolic pathway, 139
reductase (XR), 139–146, 152
transporter, 147–152
Xylose isomerase (XI)
expression, 268, 269
Piromyces sp.E2, 269, 271
Xylulokinase (XK), 139, 143, 268
Xylulose 5-phosphate (X5P), 139

Y
Yap1p response element (YRE), 106
YCA1, 65–67
Yeast, 2, 162
Yeast adaptation, 58–62
Yeast tolerance, 2
yqhD, 215, 222
YRE. *See* Yap1p response element

Z
Zygosaccharomyces bailii, 59