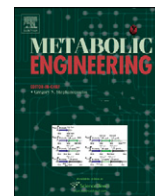




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Review

A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation

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ABSTRACT

Metabolites, substrates and substrate impurities may be toxic to cells by damaging biological molecules, organelles, membranes or disrupting biological processes. Chemical stress is routinely encountered in bioprocessing to produce chemicals or fuels from renewable substrates, in whole-cell biocatalysis and bioremediation. Cells respond, adapt and may develop tolerance to chemicals by mechanisms only partially explored, especially for multiple simultaneous stresses. More is known about how cells respond to chemicals, but less about how to develop tolerant strains. Aiming to stimulate new metabolic engineering and synthetic-biology approaches for tolerant-strain development, this review takes a holistic, comparative and modular approach in bringing together the large literature on genes, programs, mechanisms, processes and molecules involved in chemical stress or imparting tolerance. These include stress proteins and transcription factors, efflux pumps, altered membrane composition, stress-adapted energy metabolism, chemical detoxification, and accumulation of small-molecule chaperons and compatible solutes. The modular organization (by chemicals, mechanism, organism, and methods used) imparts flexibility in exploring this complex literature, while comparative analyses point to hidden commonalities, such as an oxidative stress response underlying some solvent and carboxylic-acid stress. Successes involving one or a few genes, as well as global genomic approaches are reviewed with an eye to future developments that would engage novel genomic and systems-biology tools to create altered or semi-synthetic strains with superior tolerance characteristics for bioprocessing.

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1. Introduction

1.1. Biofuels and chemicals from biomass, and the importance of strain tolerance to chemicals

The development of technologies for the production of fuels and chemicals from renewable biomass sources has been on the scientific and technological agenda of our nation for over 35 years, but never quite with the urgency of the last few years. Biomass is a carbon-neutral renewable resource for the production of biofuels and biologically produced chemicals and materials, thus leading to the widely-discussed concept of the biorefinery (Blanco-Rosete and Webb, 2008; Demirbas, 2009; Gibbons and Hughes, 2009; Rude and Schirmer, 2009; Yazdani and Gonzalez, 2007; Zheng et al., 2008). Although some success has been achieved thus far, scientific advances are expected to increase

efficiency significantly, and there is increased optimism that cost-efficient production of biofuels (Stephanopoulos, 2007), materials and chemicals from lignocellulosic biomass will be widespread within 15 years. The list of chemicals that can be in principle produced from renewable resources is large and includes simple and complex carboxylic acids and alcohols, hydrocarbons, and diesel biofuels (Blanco-Rosete and Webb, 2008; Demirbas, 2009; Gibbons and Hughes, 2009; Rude and Schirmer, 2009; Yazdani and Gonzalez, 2007; Zheng et al., 2008).

So far, biofuel production has mainly focused on ethanol, which comprises 99% of the total biofuel consumption in the US (Rao et al., 2007). Butanol is also considered as an attractive biofuel, as it exhibits superior chemical properties in terms of energy content, volatility, and corrosiveness (Lee et al., 2008). Several other oxygenated organic molecules (such as non-fermentative alcohols, including *i*-butanol (Connor and Liao, 2009; Rude and Schirmer, 2009)) that can be produced from biomass can serve as biofuels, as well. Other biofuels include biologically produced hydrocarbons deriving either from isoprenoid or fatty-acid metabolism or microbial diesel fuels (Rude and

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Schirmer, 2009). Although it is still unclear if one or more such molecules will dominate markets in the future, the fundamental features of a biomass-to-biofuels or biomass-to-chemicals processes are common. Biomass is collected and treated to release the tangled lignin–cellulose fiber via ammonia explosion, weak acid boiling, or steam treatment (Hahn-Hagerdal et al., 2006). The suspension is then digested by cellulolytic enzymes that hydrolyze the hemicellulosic and cellulosic biomass to 5- and 6-carbon sugars, which can then be fermented by alcohol producing microorganisms such as *Escherichia coli* (Ingram et al., 1987), *Clostridium acetobutylicum* or other solventogenic *Clostridia* (Lee et al., 2008; Paredes et al., 2005), *Saccharomyces cerevisiae* (Rude and Schirmer, 2009), and *Zymomonas mobilis* (Stephanopoulos, 2007). Two common features of all such processes are: (1) the presence of molecules as a result of biomass pretreatment that may be inhibitory to the fermentation process for the production of the desirable molecule(s) and (2) the inhibitory nature of the desirable product (such as ethanol or butanol, other alcohols, hydrocarbons, succinate, butyrate and other carboxylic acids) or bioproducts. Accumulation of products during a fermentation, whether desirable or not, can be toxic to the biocatalysts, inhibiting cell growth or resulting in death. Similarly, toxic contaminants in biomass-hydrolysate substrates (Almeida et al., 2007; Martin et al., 2007; Rudolf et al., 2007) can inhibit cell growth and product formation. Inhibition from such chemicals typically limit product titers, affects fermentation performance and operational options (continuous vs. batch or fed-batch), and profoundly impacts process economics.

1.2. Bioremediation and whole-cell biocatalysis also benefit from tolerant strains

Bioremediation involves the conversion of toxic chemicals into benign or less toxic chemicals by biological means and typically by employing one organisms or a consortium of microorganisms (Bustard et al., 2000, 2002; Gupta et al., 2006; Pandey et al., 2009; Zhao and Poh, 2008). The effectiveness of these microorganisms in degrading toxic chemicals depends on their natural, selected or engineered tolerance to the chemicals present during the remediation process.

Whole-cell biocatalysis in two-phase systems containing an organic phase (typically solvent) has a broad spectrum of applications for the production of specialty or fine chemicals (Heipieper et al., 2007; Neumann et al., 2006; Sardesai and Bhosle, 2004). Two-phase systems are employed in order to solubilize reactants and/or products but also to deal with the problem of toxic substrates and/or products. The organic-solvent phase frequently serves as a means to reduce the concentration of a toxic chemical from the aqueous phase. The solvent used in these two-phase systems is typically somewhat soluble in the aqueous solution, and thus the organism or organisms employed must be tolerant to such solvents, as well.

In summary, from biorefinery and biofuel production to bioremediation and whole-cell biocatalysis, development of strains with superior tolerance characteristics to specific chemicals and general stressful bioprocess conditions is an important and widely recognized goal.

1.3. Chemical toxicity and tolerance are complex, multigenic phenotypes

As detailed below, tolerance of microorganisms to chemicals is a complex, multigenic trait and is affected by several process parameters such as pH, temperature, osmotic pressure, other small or large molecules, and pressure. Indeed, if one takes the

core and most-widely examined problem of developing ethanol-tolerant strains, much effort has been based on the concept that ethanol (and more broadly solvent) tolerance can be controlled by a single gene, or a few genes. Yet, the tolerant phenotype is the result of a several simultaneous mechanisms of action, including molecular pumps, changes in membrane properties, changes in cell wall composition, altered energy metabolism, and changes in cell size and shape. These and related mechanisms are apparently independent from each other and involve genes or gene clusters widely dispersed on the chromosome or located on plasmids (Bernal et al., 2007; Isken and de Bont, 1998; Kivistik et al., 2006; Kobayashi et al., 2001; Neumann et al., 2005; Nikaïdo and Zgurskaya, 1999; Phoenix et al., 2003; Ramos et al., 2002, 1997; Volkens et al., 2006; Weber and de Bont, 1996; Wei et al., 2001).

1.4. Common chemicals and organisms encountered in biorefinery and biofuel bioprocesses

Typical or expected bioprocess-based metabolites include alcohols (e.g., ethanol, butanols and derivatized butanols, pentanols, hexanol, propanediol, butanediols), carboxylic acids (e.g., succinate, butyrate, acetate, propionate), aldehydes, ketones and hydrocarbons, and this list is ever expanding. The number of carbon substrates is also increasing as more complex carbohydrates from primary sources or from wastes are considered for the development of novel processes. They include the whole spectrum of 5- and 6-carbon sugars, glycerol, carboxylic acids (acetate, propionate), celluloses and xylans and their hydrolysates, starches, carbohydrates from corn or sugarcane refineries, and substrate impurities (such as byproducts from biomass hydrolysates). While most sugars and starches are not inhibitory to cells, most other substrates and substrate impurities can be toxic to cells.

Organisms employed in biorefinery and biofuel bioprocessing include yeast (*S. cerevisiae*, one of the major workhorses of modern biotechnology, but also other *Saccharomyces* strains, *Kluyveromyces marxianus*, *Pachysolen tanophilus*, *Sheffersomyces stipitis* (Gibbons and Hughes, 2009)) and other fungi (such as species in the genus *Aspergillus*, and *Gliocladium roseum* (Strobel et al., 2008), which is receiving attention recently in the context of microbial hydrocarbon production), *E. coli*, a major workhorse of modern biotechnology, and several other Gram-negative (Gram⁻) bacteria such as *Z. mobilis* (Antoni et al., 2007), *Mannheimia succiniciproducens* and *Actinobacillus succinogenes* (Kim et al., 2007). The list of Gram⁻ bacteria becomes much larger when considering applications in whole-cell biocatalysis and bioremediation, and would include organisms of the genus *Pseudomonas* such as *P. putida*. Among Gram-positive (Gram⁺) bacteria, the list of species of importance to biorefinery and biofuel processes, as well as to biocatalysis, includes organisms in the genera *Bacilli*, *Clostridia* and *Lactobacilli*. Among Gram⁺ organisms of importance to bioremediation processes are species of the genus *Deinococcus*, and notably *Deinococcus radiodurans*, which exhibits remarkable resistance to ionizing radiation (Cox and Battista, 2005) and is an essential microbe for remediation of sites contaminated with nuclear wastes.

2. Solvent stress, toxicity and tolerance

2.1. Classes of solvents

Solvents constitute a very wide class of molecules, both organic and inorganic. In the context of molecules produced by cells as useful or undesirable metabolites, or for applications in

bioremediation and whole-cell biocatalysis, the most significant classes of solvents are organic molecules: alcohols, aldehydes, hydrocarbons, and their halogenated derivatives. Toxicity to some of these molecules or classes of molecules has been examined mostly by classical reductionist approaches over the last 20–40 years. The model, and best understood, cases are ethanol toxicity in *E. coli* and *S. cerevisiae*, hydrocarbon toxicity in *P. putida*, and butanol toxicity in *C. acetobutylicum*.

2.2. Overview of cellular responses to solvents, mechanisms of toxicity and tolerance

Cellular responses to solvent stress have been examined quite extensively in model organisms over the last 40+ years by engaging physiological and molecular tools and reductionist approaches. This information has been reviewed in several older and recent reviews (Isken and de Bont, 1998; Ramos et al., 2002; Sardesai and Bhosle, 2002; Sikkema et al., 1995). In the last 10 years, genomic tools (microarray-analysis-based transcriptomics, proteomics, and metabolomics) have been increasingly used to examine the stress response to various solvents and other chemicals (Alsaker et al., 2010, 2004; Borden and Papoutsakis, 2007; Brynildsen and Liao, 2009; Ramos et al., 2009; Rutherford et al., 2010; Tomas et al., 2004; Volkers et al., 2009, 2006). Similarly, genomic tools have been employed to interrogate mechanisms of toxicity and tolerance, and develop strategies for generating and assessing tolerant strains. Several studies (e.g., Isken and de Bont, 1998; Kobayashi et al., 1998; Ramos et al., 2002; Sardesai and Bhosle, 2002; Vanbogelen et al., 1987) have shown that solvents affect cells by

- (a) damaging and denaturing biological molecules, including the unfolding of proteins; DNA and lipid damage by oxidative and related mechanisms; and RNA unfolding and degradation. These effects may elicit severe stress responses and cell death; and
- (b) imparting biophysical changes to cell membranes, thus affecting membrane processes, including energy generation and transport.

Cells respond to these effects pleiotropically, such as by

- (i) inducing general and specific stress responses which engage major or specialized components of the stress response system (heat-shock (HSP) and related stress proteins);
- (ii) inducing metabolic and transport-based detoxification mechanisms, including processes to metabolize the toxic chemical to a less toxic one; induction and employment of transporters/molecular pumps to expel the toxic chemicals; and alterations in biosynthetic programs to produce metabolites, such as certain amino acids and sugars, that counteract the toxic effects;
- (iii) eliciting longer-term adaptive responses to overcome solvent toxicity, such as by altering biosynthetic and morphogenetic/differentiation programs for counteracting solvent toxicity, including alterations in membrane and/or cell-wall composition; and
- (iv) inducing complex transcriptional and protein-level changes which at present are merely cataloged and generally little understood programmatically or systematically.

Details are provided in the discussion below, which is organized in sections based on mechanisms of cellular responses or adaptation to chemical stressants, and Fig. 1 provides a visual summary for these.

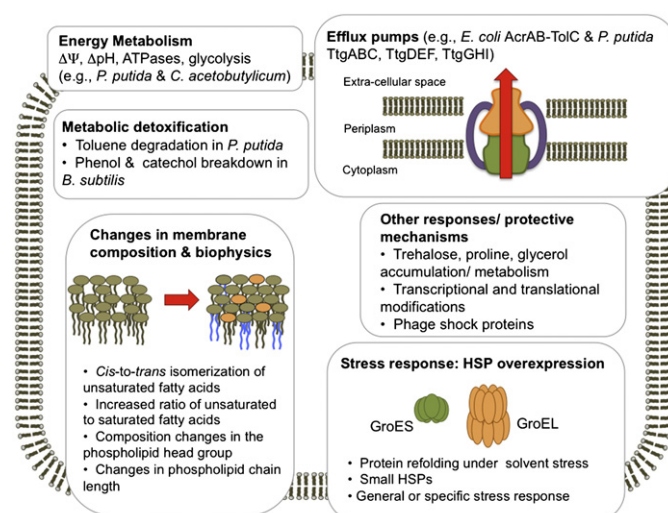


Fig. 1. Cellular responses to toxic solvents that might impart tolerance. Toxic solvents induce numerous responses to combat stress and enhance survivability. This figure summarizes pictorially important responses that might provide tolerance to Gram-negative and/or Gram-positive prokaryotes and/or yeast cells. Cell membrane composition and biophysics change to counteract the fluidizing effects of solvents, including the short term *cis-to-trans* isomerization of phospholipids, and the long term changes in the ratio of unsaturated to saturated fatty acids. Molecular efflux pumps (here shown spanning the periplasm of a Gram-negative cell) expel solvents from the cytoplasm into the extracellular space in order to detoxify the cytoplasm. Disrupted membrane processes, such as the protonmotive force and associated energy production, elicit changes in cellular metabolism for higher ATP production necessary for survival and cellular repair processes. HSP stress proteins assist in protein folding and re-folding and prevent aggregation under chemical stress. Catabolic detoxification removes toxic compounds by converting them into less harmful chemicals. Other transcriptional and translational responses may enhance viability under stress by leading to production of osmolites like trehalose, or by upregulating specialized stress systems, such as the phage-shock protein system.

2.3. Impact on membranes and membrane functions

Cell membranes are composed of a phospholipid bilayer interspersed with proteins. In addition to providing structural integrity and maintaining a barrier to the extracellular environment, they facilitate transport in and out of the cell and are responsible for signal transduction, communication, and energy production. The effects of solvents, particularly alcohols, aldehydes and lipophilic compounds, on cell integrity and membrane composition have been studied extensively (Bernal et al., 2007; Buttke and Ingram, 1978, 1980; Ingram, 1976; Ingram et al., 1980; Inoue and Horikoshi, 1989; Sikkema et al., 1994, 1995). Organic solvents intercalate in the phospholipid bilayer of membranes, thus disrupting the membrane electrochemical potential ($\Delta\Psi$) and the proton gradient (ΔpH), displacing proteins, and more generally inhibiting membrane functions (Sardesai and Bhosle, 2002). The fluidity of the membrane increases, and, when the solvent concentrations are high, the cells cannot actively maintain cellular homeostasis and this may lead to cell death.

It is widely accepted that the toxicity of a solvent is well correlated with the partition coefficient ($\log P$) of the solvent (Inoue and Horikoshi, 1989). $\log P$ is defined as the partition of the solvent in an equimolar mixture of octanol and water. High polarity solvents have a low $\log P$, and such solvents accumulate in cell membranes to high concentrations, severely damaging or killing cells. Thus, low $\log P$ values are indicative of highly toxic solvents. However, the tolerance level of each organism to various solvents (i.e., the $\log P$ values of solvents it can tolerate) is strain specific. The $\log P$ value of most toxic solvent that a microorganism

can survive is designated as the index value: thus, a microorganism can survive in solvents with higher $\log P$ than its index value (Sardessai and Bhosle, 2002).

Gram⁻ bacteria possess an additional cell membrane (an outer membrane) and a periplasmic space, and these cellular structures might be important as to of the mechanisms they engage to deal with solvent toxicity. Gram⁺ bacteria have a single membrane, but their cell wall contains more peptidoglycans, which may protect cells from solvent toxicity (Sardessai and Bhosle, 2002). Much less is known about the impact of solvents and toxic chemicals on Gram⁺ organisms such as *Lactobacilli* and *Clostridia* (Borden and Papoutsakis, 2007).

Since solvents alter membrane fluidity and decrease the stability of the phospholipid bilayer, cellular responses aim to compensate for this by changing the rate of biosynthesis and composition of phospholipids. Upon initial stress, cells express genes (such as the *cis-to-trans* isomerase in *P. putida*) to increase the isomerization of *cis-to-trans* unsaturated fatty acids and counteract the increasing membrane fluidity (Loffeld and Kewelloh, 1996; Ramos et al., 2002; Weber et al., 1994). A longer-term response is to change the ratio of saturated-to-unsaturated fatty acids present in the membrane, an effect that is also elicited by increased temperature (Ingram, 1977). The response depends on the Carbon-chain length of the solvent used. For example, when exposed to short-chain (C2–C4) alkanols, *E. coli* increases the unsaturated fatty acid concentration of its cell membrane. However, long-chain (C5–C9) alkanols result in an increase in the saturated fatty acid content (Weber and de Bont, 1996). Different species also react differently to the effects of solvents such as ethanol, with *E. coli* (Ingram, 1976) and *S. cerevisiae* (Beaven et al., 1982) increasing the concentration of unsaturated fatty acids in the lipid composition, and other organisms like *Bacillus subtilis* (Rigomier et al., 1980) and *C. acetobutylicum* (Borden and Papoutsakis, 2007; Lepage et al., 1987) increasing the content of saturated fatty acids (Weber and de Bont, 1996). The proposed model is that membrane functions are protected by increasing the concentration of unsaturated fatty acids to minimize the effects of solvents on the bilayer structure, or by increasing the saturated fatty acids to restore order in the membrane (Weber and de Bont, 1996). Analogous to the prokaryotic response, yeast cells also adjust the composition of membrane fatty acids to ameliorate the impact of solvents on membrane fluidity and functions (Ding et al., 2009; Zhao and Bai, 2009). There is some evidence that yeast cells also alter the composition of the cell wall (Zhao and Bai, 2009), which is considerably more complex and much stronger than prokaryotic cell walls. The stronger cell wall of yeast cells is frequently assumed to be the reason that yeast cells are typically more tolerant to solvent stress, although, as discussed below, this is not always the case.

2.4. Efflux pumps

In Gram⁻ organisms like *E. coli*, *P. aeruginosa* and *P. putida*, proteins of the multi-drug efflux-pump family and other transporters (Box 1) play an important role in solvent tolerance (Aono et al., 1995). Little is known about the impact of efflux pumps on solvent tolerance in Gram⁺ organisms like *Bacilli* and *Clostridia* (Sardessai and Bhosle, 2002). Efflux pumps are made up of three components spanning both the inner and outer membranes: an inner membrane transporter that uses ATP derived from the membrane proton motive force to enable export, a membrane fusion protein (MFP, located in the periplasm that complexes with the other proteins generating a channel), and an outer membrane protein (OMP, which facilitates efflux outside the cell) (Ramos

et al., 2002). Thus, in Gram⁻ prokaryotes, efflux pumps enable the transport of chemicals (including solvents, antibiotics and other drug molecules) across the inner and outer membranes and into the media (Zgurskaya and Nikaido, 1999).

In *E. coli*, the AcrAB-TolC system acts as an efflux pump, with AcrB as the inner membrane transporter acting as a peristaltic pump, AcrA being the MFP and TolC being the OMP (Koronakis et al., 2000; Pos, 2009; Ramos et al., 2002; Seeger et al., 2006). Expression of *acrAB* and related genes is modulated by the transcriptional regulators *marR*, *marA*, *soxR*, and *soxS* (Asako et al., 1997; Oethinger et al., 1998; White et al., 1997). DNA microarray analysis was used to examine the *marA* regulon by analyzing the effect of constitutive MarA expression: 60 chromosomal genes belonging to a variety of functional groups were affected including genes of the *soxRS* regulon. Overexpression of *marA*, *robA* and *soxS* increased the production of AcrA and TolC, resulting in higher cyclohexane tolerance due to increased activity of the efflux pump (Aono et al., 1998). This agrees well with reports that *marA* overexpression increases *E. coli* organic solvent tolerance (Asako et al., 1997; White et al., 1997). In contrast, and to further support the relationship between the *acrAB* operon and solvent tolerance, deletion of *acrAB* resulted in loss of tolerance to both n-hexane and cyclohexane (White et al., 1997). Furthermore, a mutation in *marR*, the gene that codes for the repressor of the *mar* operon (containing the *marR*, *marA* and *marB* genes), resulted in increased solvent tolerance, further emphasizing the importance of the efflux pump (Aono, 1998). Other such pumps include the AcrEF and TolC efflux pump in *E. coli* (Jellen-Ritter and Kern, 2001), the Pdr12 transporter in *S. cerevisiae* (Piper et al., 1998), and the TtgABC, TtgDEF and TtgGHI efflux pumps in *P. putida* (Ramos et al., 2002; Rodriguez-Herva et al., 2007; Rojas et al., 2001). The Rob protein (a member of the AracC-XylS family) regulon was also related to solvent tolerance in *E. coli* (Bennik et al., 2000). In *P. aeruginosa*, antibiotic and solvent tolerance have been linked to the action of several efflux pumps: MexA–MexB–OprM, MexC–MexD–OprJ, and MexE–MexF–OprN (Li et al., 1998). Similar efflux systems have been found in *P. putida* strains and were shown to impact solvent tolerance (Hirayama et al., 1998; Isken and de Bont, 1996; Kieboom et al., 1998; Ramos et al., 1998). *P. putida* S12 contains the SprABC efflux system, which was shown to impart toluene tolerance when expressed in a non-tolerant strain (Kieboom et al., 1998). The SprABC efflux system is homologous to the TtgGHI system in *P. putida* DOT-T1E (Sun and Dennis, 2009), and is regulated by the *srpR* and *srpS* genes.

2.5. Heat-shock proteins (HSPs) and solvent tolerance

The cellular stress response system (Box 2) utilizes HSPs, also called molecular chaperones, and play an essential role in the synthesis, transport, folding, and degradation of proteins. HSP expression is induced by a very wide of stressants including solvents and other toxic chemicals in virtually all organisms that have been examined by traditional or global genomic approaches (e.g., Alexandre et al., 2001; Alsaker et al., 2010, 2004; Brynildsen and Liao, 2009; Polen et al., 2003; Rossignol et al., 2003; Rutherford et al., 2010; Tam et al., 2006; Tomas et al., 2004). There is however rather limited literature regarding the use of HSP genes/proteins to generate solvent-tolerant strains. Expression of HSP genes and other stress genes is regulated by specialized transcription and sigma factors (Box 2) through a variety of mechanisms, most still largely not understood. Among these sigma factors, the most important one for general stress is σ^S in Gram⁻ organisms (such as *E. coli*) and σ^B in *Bacilli* and other (but not all) Gram⁺ organisms (Box 2). These will be discussed in detail in the following sections.

Box 1: Molecular pumps, multidrug resistance and solvent tolerance.

Microorganisms contain unidirectional efflux systems, known as molecular pumps, which actively expel chemicals from the cytoplasm to the extracellular space and protect cells from the harmful effects of these chemicals. These pumps are involved in multidrug resistance (MDR) and, because of their substrate promiscuity and broad specificity, they can contribute to solvent tolerance. There are five families of these pumps: (i) the **ABC** family (containing an ATP-binding cassette), (ii) the major facilitator superfamily (**MFS**), (iii) the small multidrug resistance (**SMR**) family (iv) the multidrug and toxic compound exporters (**MATE**) and (v) the resistance-nodulation-division (**RND**) family (Higgins, 2007; Krulwich et al., 2005; Segura et al., 1999).

All transporters utilize energy (ATP or electrochemical (H^+/Na^+)) to shuffle chemical (drugs) against a concentration gradient outside of the cell in an antiport mechanism. ABC transporters utilize ATP energy to transport inorganic ions, amino acids, hydrocarbons, polypeptides or hydrophobic compounds and participate in multidrug resistance (Higgins, 2007). MFS transporters differ in structure from ABC transporters and utilize the proton motive force as their energy source (Higgins, 2007). SMR transporters, found in prokaryotes, derive energy from the proton motive force and generate a simple channel of transmembrane α -helices to facilitate transport (Higgins, 2007). MATE transporters utilize energy from the H^+ or the sodium motive force (Na^+) for antiport transfer of drugs (Krulwich et al., 2005). RND transporters utilize energy from the proton motive force to remove antibiotics, bile salts and acids, steroids, and solvents (Blair and Piddock, 2009; Pos, 2009).

In Gram-negative (Gram⁻) bacteria, RND transporters constitute the efflux pumps associated with solvent tolerance (Ramos et al., 2002). Examples include the AcrAB-TolC efflux pump in *E. coli*, and the TtgABC and TtgDEF pumps in *P. putida* (Ramos et al., 2002). In *E. coli*, the RND efflux pumps are comprised of an inner membrane pump (AcrB) and an outer membrane factor or channel (TolC) linked together by an adaptor membrane fusion protein (AcrA) that spans the periplasmic space (Symmons et al., 2009). The complete efflux pump (Fig. 1) forms the channel for multidrug transport that removes solvents from the cell cytoplasm.

Transporters have an integral role in maintaining pH homeostasis. The Tet(L) transporter in *B. subtilis* and the MdfA efflux pump in *E. coli* are both associated with MDR and facilitate alkali tolerance (Krulwich et al., 2005). C-4-dicarboxylate carriers, such as the DctA and Dcu in *E. coli*, facilitate the uptake and efflux of organic acids such as succinate and citrate (Janausch et al., 2002). Although these pumps have not been directly linked to tolerance in bacteria, they may play an important role in carboxylic acid stress during fermentation. This is particularly apparent in yeasts, where carboxylic acid transport has been studied more extensively (Casal et al., 2008). Notably, the carboxylate ABC efflux pump Pdr12p in *S. cerevisiae* is involved in C1–C7 monocarboxylic-acid resistance (Holyoak et al., 1999). Furthermore, Pdr12p is shown to confer resistance to acids obtained from the metabolism of leucine, isoleucine, valine, phenylalanine and tryptophan (Hazelwood et al., 2006). Molecular pumps have a role in acid tolerance in yeasts, and it is possible that they play an analogous role in bacteria, as well.

Overexpression of heat-shock proteins was shown to impart higher survivability under stress. In *C. acetobutylicum*, *groESL* overexpression (Tomas et al., 2003) reduced growth inhibition due to butanol stress by 85%, and prolonged cell metabolism by up to 2.5 times. Most importantly from a bioprocessing perspective, solvent titer production increased by 40% relative to the wild type strain (Tomas et al., 2003). In follow-up work, it was demonstrated that when cells are stressed by butanol, *groESL* overexpression results in increased expression of several other HSP genes, including *dnaKJ*, *hsp18*, *hsp90* (Tomas et al., 2004).

Increased solvent tolerance due to HSP overexpression was also demonstrated in other organisms, including *Lactococcus lactis*, *Lactobacillus paracasei* NFBC 338 (Desmond et al., 2004), *Lactobacillus plantarum* (Fiocco et al., 2007) and *S. cerevisiae* (Vianna et al., 2008). In *P. putida*, *groESL* upregulation was observed during solvent stress (Segura et al., 2005; Volkers et al., 2006), but it was not conclusively demonstrated that the *groESL* upregulation results in increased solvent tolerance.

In the psychrophile *B. psychrosaccharolyticus*, it was observed that solvent stress from ethanol, isopropyl alcohol (IPA), acetone, benzene and butanol induced the upregulation of *hsp33* (Kang et al., 2007). Overexpression of the *B. psychrosaccharolyticus* Hsp33 in *E. coli* increased the *E. coli*'s tolerance to IPA, demonstrating that a psychrophilic protein is functional at higher temperatures and confers a tolerant phenotype (Kang et al., 2007). Recently, the overexpression of a chaperonin and its co-factor prefoldin from the archaeum *Pyrococcus horikoshii* OT3 in *E. coli* was shown to result in higher n-hexane and octane tolerance (Okochi et al., 2008).

As reviewed (Piper, 1995), sub-lethal heat and ethanol stresses induce virtually identical responses in *S. cerevisiae*, and a role for Hsp30 and Hsp104 in ethanol stress-response and tolerance identified. Among the large number of upregulated HSPs, there are enzymes with anti-oxidant properties, thus suggesting that both heat and ethanol stresses are inducing some oxidative

damage. In contrast to these conclusions, a recent genome-wide screening (using a *S. cerevisiae* collection of deletion mutants) to identify genes that impart sensitivity to heat, alcohol (ethanol, methanol, and 1-propanol), osmotic stress, and oxidative stress, did not identify any HSP genes (Auesukaree et al., 2009). These screens look for sensitivity to a stressant in the absence of a gene, and are not equivalent to screens for tolerance. Moreover, some HSP proteins work together with partners, co-chaperons or co-factors (like in the GroES and GroEL HSP system; Box 2), and thus, no single gene deletions are likely to capture the impact of some at least HSP proteins on solvent tolerance. Yet, in another recent study, where another *S. cerevisiae* deletion collection was used to screen for genes that affect sensitivity to 6% ethanol, among the various genes identified was the co-chaperone GimC (van Voorst et al., 2006).

These studies demonstrate that HSPs are a line of defense against cell damage and death due to solvent stress and can enhance solvent tolerance. The most likely mechanism of protection is by stabilizing or by refolding proteins which are crucial for cell metabolism and survival, and which are sensitive to solvent stress.

2.6. Detoxification by metabolic breakdown

Catabolism of chemicals during bioprocessing might not be desirable because the product (for example solvent production in ethanologenic *E. coli* or butanol produced by solventogenic *Clostridia*) might be the toxic chemical that is degraded. However, detoxification might prove useful in bioprocessing using biomass hydrolysates or in bioremediation applications. For the former, an important example would be the case whereby substrate impurities are toxic, but a strain has been endowed by selection or metabolic engineering to convert the impurities to non-toxic chemicals thereby removing the toxicity. Another example would

Box 2: Heat shock proteins, other stress proteins, and specialized stress regulons.

The cellular stress response utilizes heat-shock proteins (HSPs), which are molecular chaperones assisting in protein folding and refolding of proteins damaged by chemical and other stress (Guisbert et al., 2008; Han et al., 2008; Kultz, 2005; Lund, 2009), but also several other non-HSP stress proteins. Cells also engage specialized, for various types of stresses, sigma factors to transcribe or upregulate their regulons for ameliorating stress-induced cell damage. Cells contain multiple lines of defense against protein aggregation, DNA and RNA damage, as well as against membrane and organelle damage. The interplay of HSP proteins with other proteins (involved in stress as well as energy generation and core metabolism) facilitates protection from and tolerance to chemical and other stresses.

Stress systems have been most extensively studied for *Escherichia coli*, the Gram-negative (Gram⁻) model organism. HSPs have been classified based on approximate size (or the size of the prototypical HSP protein of a family), and they include DnaK (Hsp70), DnaJ (Hsp40), GrpE, GroEL (Hsp60), GroES (Hsp10), the Clp family (Hsp100 proteins), as well as many small stress proteins (Chung et al., 2006; Han et al., 2008; Horwich et al., 2006; Lund, 2009; Qiu et al., 2006). Under normal conditions, the major HSP proteins are responsible for folding nascent proteins, translocating proteins across membranes, assembling or disassembling oligomeric protein structures, and facilitating the degradation of unstable proteins to prevent protein aggregation (Tomas et al., 2003). Under stress conditions, their role is to prevent aggregation and assist in protein refolding of damaged proteins (Tomas et al., 2003). The chaperonin GroEL (Fig. 1) is made up of two rings of seven subunits that create a hollow barrel where misfolded proteins can enter for refolding. GroES caps the opening, creating a favorable environment where misfolded proteins are refolded using energy from ATP hydrolysis (Ranson et al., 1998). DnaK is characterized by a “brick-shaped” domain with a hole through which proteins to be folded run (Tomas et al., 2003). The GroEL-GroES complex in *E. coli* has been implicated in the folding or refolding of approximately 300 proteins, thus rendering it an essential system for cell survival (Lund, 2009). DnaK binds to exposed hydrophobic regions of proteins and acts in cooperation with DnaJ and GrpE to fold partially folded or unfolded proteins utilizing ATP (Zhu et al., 1996).

Hsp33 is another important chaperone which is upregulated during redox-, heat- or solvent-stress in *E. coli* (Graf and Jakob, 2002; Hoffmann et al., 2004). At elevated temperatures and oxidative stress, DnaK is inactivated by the lack of intracellular ATP; thus, Hsp33, whose actions is ATP independent, takes over the role of minimizing protein aggregation (Winter et al., 2005).

In Gram positive (Gram⁺) bacteria, HSPs have been classified based on their classification in the model organism *Bacillus subtilis*. There are four classes of HSPs in *B. subtilis*. Class I HSP genes include those coded by the *dnaK* and *groESL* operons. Class I heat-shock operon expression is regulated by the repressor protein HrcA (Tomas et al., 2004). The *dnaK* and *groESL* operon structures in *C. acetobutylicum* are nearly identical to those in *B. subtilis* (Bahl et al., 1995) and have been shown to share the same regulatory mechanisms. The *groE* operon consists of two genes, *groES* and *groEL* (Narberhaus and Bahl, 1992), while the *dnaK* operon consists of seven genes, *orfA*, *grpE*, *dnaK*, *dnaJ*, *orfB*, *orfC*, and *orfD* (Narberhaus and Bahl, 1992; Rungeling et al., 1999). In *Bacilli*, class II HSP genes include stress response genes regulated by the secondary and stress-specific σ^B sigma factor (Helmann et al., 2001). The σ^B system has not been identified in *Clostridia*, which thus lack the Class II HSP proteins. Class III HSPs include *clp* genes and are regulated by the CtsR repressor (Derre et al., 1999). ClpB, ClpA and ClpX are approximately 100 kDa in size and are involved in tolerance to extreme temperatures (Horvath et al., 2008). Class IV contains genes (such as *ftsH*), which are upregulated during heat or other stress, but are not regulated by HrcA, σ^B , or CtsR (Tomas et al., 2004). Several Classes I, III and IV genes/proteins have been recently identified in clostridia based on gene expression patterns in response to chemical (butanol, butyrate, and acetate) stress (Alsaker et al., 2010). In Gram⁺ bacteria, the role of the major HSPs in solvent and stress response is similar to the role of their orthologous counterparts in Gram⁻ organisms.

Most organisms contain specialized sigma factors which they engage to respond to general or specialized stress. The σ^B system discussed above for *Bacilli* is a well-studied case, but again, such specialized stress sigma have been most extensively studied in *E. coli* and other Gram⁻ bacteria. In *E. coli*, there are six sigma factors associated with stress response including σ^{54} (σ^N nitrogen depletion), σ^{32} (σ^H heat shock), σ^{24} (σ^E extracytoplasmic stress), σ^{28} (σ^F flagella synthesis and chemotaxis), σ^{38} (σ^S starvation and general stress response), and σ^{19} (citrate-dependent iron transport) (Chung et al., 2006; Loewen et al., 1998). Sigma factor σ^S , encoded by *rpoS*, is the major regulator of the general stress response and affects the transcription of over 10% of the genes in *E. coli* (Weber et al., 2005). It has an extensive overlap with other global regulons (e.g., the cAMP regulon), interacts with the major transcription factor Lrp, and impacts not only stress physiology, but more broadly cell physiology under non-optimal growth conditions (Weber et al., 2005). Its regulon is far from rigid and exhibits complex internal control as demonstrated by the pattern of expression of genes involved in acid resistance (Weber et al., 2005). In *Pseudomonas aeruginosa*, RpoS controls almost 800 genes in the stationary phase of cell growth, including more than 40% of all quorum-sensing controlled genes (Schuster et al., 2004).

In *S. cerevisiae*, the heat-shock factor *hsf1* regulates the stress response of the organism, which includes HSPs involved in solvent tolerance (Ding et al., 2009; Piper, 1995). Ethanol stress results in nuclear accumulation of Hsp70, which suggests that protein repair is necessary for tolerance (Quan et al., 2004). Hsp30 is induced under ethanol, heat shock and weak organic acid stress (Seymour and Piper, 1999). Similarly, Hsp12 is associated with cold, oxidative, and ethanol stress (Pacheco et al., 2009). However, it must be noted that although HSP overexpression is typical under solvent stress, the exact mechanism of tolerance in yeast cells is not yet known (Ding et al., 2009).

be a toxic intermediate which could be converted either to a final product (such as the case of conversion of butyrate to butanol in clostridial fermentations (Papoutsakis, 2008)) or to a non-toxic chemical. In bioremediation, in addition to the target chemical to be remediated, additional chemicals might be present in the contaminated soils or waters, and such chemical may be toxic to the cells or may inhibit the detoxification of the target chemical.

The widely examined case of degradation of a toxic chemical is toluene degradation by *P. putida*, which exhibits tolerance to toluene, although, as discussed below, degradation is not necessary for tolerance to toluene. *P. putida* can degrade toluene employing two pathways. The TOL pathway (coded on the pWWO

plasmid of *P. putida* mt-2) enables the oxidation of toluene to benzoate and eventually to catechol (Worsey and Williams, 1975). The toluene dioxygenase (TOD) pathway of *P. putida* DOT-T1 facilitates the conversion of toluene to 3-methylcatechol (Mosqueda et al., 1999). Catechols produced by both pathways are then degraded by catechol 2,3-dioxygenases (Ramos et al., 2009). The breakdown of toluene to less toxic compounds might contribute, partially at least, to tolerance by eliminating the stressor. However, it was shown that *P. putida* strains able to metabolize toluene maintain their high solvent tolerance even when the pathway for toluene catabolism is disrupted (Mosqueda et al., 1999). This demonstrates that metabolic breakdown alone

is not responsible for the tolerance to toluene in this organism. Another example is that of n-hexane-tolerant *E. coli* strains which cannot metabolize the solvent, and thus n-hexane tolerance must be attributed to other factors (Ramos et al., 2002). It is also possible that the products of metabolism are more toxic to the cells, such as when octane is degraded to octanol (Ramos et al., 2002).

Beyond the well-known capabilities of *Pseudomonas* species, *Bacilli* also degrade an array of chemicals, including phenols and catechols, and possess catabolic pathways for degradation of aromatics (Duffner et al., 2000; Leelakriangsak et al., 2008; Tam et al., 2006). In *B. subtilis*, the monocyclic aromatic compound catechol induces the derepression of catabolite-controlled genes but this response depends on the glucose concentration in the medium (Tam et al., 2006). It was also reported that catechol induces a stress response in *B. subtilis*, including an HSP response (through the *hrcA* and *ctsR* regulons; see Box 2) and an oxidative stress response (through the *spx*, *perR*, *furR* regulons) (Tam et al., 2006). Two genes, *yfiD* and *yfiE*, coding for an internal membrane protein and a dioxygenase (sharing homology with a catechol-2,3-dioxygenase) were upregulated during catechol stress, and mutants exhibited sensitivity to the chemical (Tam et al., 2006). Overexpression of *yfiE* exhibited 2,3-dioxygenase activity (Tam et al., 2006), thus, suggesting that catechol degradation confers tolerance.

To summarize, although metabolic detoxification may not alone explain tolerance to chemicals for some organisms, nonetheless, degradation of a toxic chemical into a non-toxic one would certainly remove its inhibitory effects on an organism. Thus, it is possible to enhance the tolerance of an organism by incorporating metabolic pathways that degrade stress-inducing chemicals. For example, introduction of a novel pathway in a microorganism to break down the toxic byproducts of lignocellulosic degradation (such as furfural or other furans; discussed below in detail) would enable better growth and metabolism of the cells on the hydrolysate sugars. As another example, overexpression of catabolic genes, such as the *yfiE* from *B. subtilis* or the toluene degradation pathway from *P. putida*, in suitable hosts can be used for bioremediation of heavily polluted areas. In such applications, chemical degradation can be a desirable property that can be optimized by metabolic-engineering-based strain development.

2.7. A class of stress-like phage proteins that are involved in chemical tolerance

The phage-shock protein (Psp) (Engl et al., 2009; Jovanovic et al., 2009) of enterobacteria is induced in response to a damaged inner membrane, and is thought to help maintain the membrane protonmotive force. In view of the fact that solvents (and also possibly certain carboxylic acids) may damage the inner membrane, an involvement of the Psp system in solvent stress and tolerance would be expected. Indeed, *E. coli* expresses the phage shock protein A (PspA) (Brissette et al., 1990) when grown under solvent stress (Kobayashi et al., 1998). PspA, an inner membrane protein, and a negative regulator of the Psp system, is induced in the presence of stress resulting from solvents such as ethanol, heat shock, hyperosmotic shock, extensive growth in the stationary phase, proton ionophores, osmolarity imbalance, or by inhibition of ATP or fatty acid synthesis (Aono, 1998; Kleerebezem et al., 1996). Although PspA is believed to help maintain the proton membrane potential and is involved in solvent tolerance, the exact mechanism of action is still under investigation (Darwin, 2005; Engl et al., 2009). PspA was studied

in vitro to show that the protein binds to phosphatidylserine- and phosphatidylglycerol-containing liposomes and restores the membrane potential by repairing the leakage of protons, through a yet unknown mechanism (Kobayashi et al., 2007). By suppressing the loss of protons, PspA allows for extended survival of the cells, and thus PspA is involved in solvent tolerance. Evidence for the involvement of phage proteins in solvent stress outside enterobacteria was recently presented for *C. acetobutylicum* (Alsaker et al., 2010).

2.8. Energy and energetic processes are crucial for dealing with solvent stress

The engagement of energy-dependent processes for dealing with solvent toxicity, such as the employment of efflux pumps and HSP proteins, would suggest that energy generation is a crucial process in dealing with and ameliorating solvent and other chemical stress. Furthermore, with solvent-induced damage of cellular macromolecules and the need to repair or re-synthesize such molecules would anticipate increased energetic needs in response to solvent toxicity. Such evidence has been accumulating.

Energetic processes in solvent tolerance in *P. putida* have been recently reported (Neumann et al., 2006; Ray and Peters, 2008; Segura et al., 2005; Volkert et al., 2009). In a proteomic study, Segura et al. (2005) report that toluene stress induced the expression of genes involved in sugar transport, glucose catabolism, and the Krebs cycle, which suggest that the cells are trying to compensate for the high energy expenditure required to survive stress, particularly the ATP cost associated with efflux pumps. In another proteomics study, it was demonstrated that the energy supply of *P. putida* under toluene stress was heavily affected due to the disruption of the proton motive force, and, to counteract the downregulation of ATP synthase, genes involved in NAD(P)H metabolism were upregulated (Volkert et al., 2006). The increased demand for energy in cells under toluene stress was also investigated via transcriptomics: genes involved in energy production and sugar consumption were upregulated whereas those involved in sugar storage were downregulated (Volkert et al., 2009). In addition to toluene studies, the effects of the chemical stressors 2,4-dinitrophenol (DNP), pentachlorophenol (PCP), and N-ethylmaleimide (NEM) on the metabolism of *P. aeruginosa* were also investigated (Ray and Peters, 2008). These chemicals were chosen because DNP induces the heat-shock response and is a respiratory uncoupler, PCP induces the expression of stress proteins, and NEM is an oxidative stressor (Ray and Peters, 2008). At low concentrations of DNP and PCP, biomass production decreased while the rate of metabolism (i.e., substrate use) was unaffected (Ray and Peters, 2008). This suggests that the HSP response elicits the allocation of cellular resources from growth to stress management, which is in agreement with the experimental evidence for *P. putida* under toluene stress.

In a comparative study between ethanologenic *E. coli* KO11 (parent strain) and the ethanol-tolerant LY01 (mutant), it was demonstrated that genes involved in the central intermediary (13% of all genes) and energy (23% of all genes) metabolism were differentially expressed (Gonzalez et al., 2003). In yeast, while ethanol stress reduces the protein levels of the membrane ATPase (probably due to general downregulation of protein synthesis), the activity of this protein increases (Piper, 1995). This allows for the efflux of protons and energy generation to continue, which offsets the dissipation of the proton motive force brought about by ethanol stress (Piper, 1995). It was observed that in non-tolerant yeast strains grown in anaerobic conditions, the alcohol yields are elevated. This can be presumably explained by

the fact that ethanol stress increases the demand for energy and cells produce ethanol at higher levels in order to generate more ATP (Devantier et al., 2005). Gorsich et al. (2006) report that genes of the pentose phosphate pathway are induced during furfural stress, which suggests that the cells are trying to counteract the effects of decreased protonmotive force in order to maintain sufficient energy levels.

In *C. acetobutylicum*, high concentrations of butanol inhibit active nutrient transport, the membrane bound ATPase, and glucose uptake (Bowles and Ellefson, 1985); partially or completely abolish the membrane ΔpH (Bowles and Ellefson, 1985; Gottwald et al., 1984; Terracciano and Kashket, 1986) and $\Delta\mu$ (Terracciano and Kashket, 1986); and lower the intracellular pH (Bowles and Ellefson, 1985; Huang et al., 1986; Terracciano and Kashket, 1986) and ATP concentration (Bowles and Ellefson, 1985). Clearly then, the cells must expend considerably higher levels of biosynthetic energy to deal with butanol toxicity, and evidence to this effect is now accumulating. For example, transcriptomic data suggest that energy-generation processes are upregulated during butanol stress in *C. acetobutylicum* (Alsaker et al., 2010, 2004). A more recent study of multiple stress (butanol, butyrate, acetate) transcriptomic analysis coupled with ontological and pathway analysis (Alsaker et al., 2010) shows that moderate butanol stress upregulates the energy-generation machinery, sustains glucose uptake at pre-stress levels while at the same time downregulating protein biosynthesis. The latter presumably is meant to make more ATP available for HSP-based related protein-refolding processes and/or de novo biosynthetic processes aiming to alter membrane composition. Metabolic engineering of the energy-generation machinery of cells remains to be actively explored as a means to enhance tolerance.

2.9. Trehalose, glycerol, proline and other amino acids as enhancers of chemical tolerance

In *E. coli* and other Gram⁻ bacteria, in Gram⁺ organisms like *B. subtilis*, and in yeast cells (*S. cerevisiae*), the synthesis and/or uptake of so-called osmolytes or compatible solutes (such as trehalose, proline, glycine betaine, and glycerol or glycosylglycerol) is used as a means of protection against osmotic stress (Kempf and Bremer, 1998). There is accumulating evidence that some of these or similar solutes are also involved in protection against solvent stress, and possibly carboxylic-acid stress.

Trehalose, a ubiquitous disaccharide made up of two glucose molecules linked by an α,α -1,1-glycosidic linkage, is synthesized by many yeast and bacterial cells. In *E. coli*, trehalose biosynthesis is controlled (Strom and Kaasen, 1993) by the stress-specific sigma factor RpoS or σ^S (see Box 2), which is the master regulator of starvation and general stress responses. In both bacteria and yeast, trehalose accumulates to protect cells from osmotic stress, but also from heat, salt, starvation and oxidative stress, and is thus viewed as a small molecular-mass “chaperone” that protects proteins and cellular membranes from unfolding and damage (Arguelles, 2000). Accumulation of trehalose and proline in yeast cells under ethanol stress was recently firmly established (Kaino and Takagi, 2008), and their role in protecting yeast cells against ethanol and other stresses has been recently reviewed (Ding et al., 2009; Shima and Takagi, 2009). Although not yet widely explored, other amino acids (isoleucine, methionine and phenylalanine) have also been found to offer protection against ethanol stress (Ding et al., 2009). There is some evidence that trehalose can also protect bacterial cells from solvent stress, in addition to osmotic stress and stress from drying (xeroprotection). This was demonstrated for *P. putida* (Vilchez et al., 2008), whereby cells dried in the presence of trehalose had superior tolerance to many stresses

including solvent, acid and oxidative stress. It was also shown that increased trehalose production as induced by indole-3-acetate, protected *E. coli* cells from acid and oxidative stress (Bianco et al., 2006).

Similarly, glycerol accumulation has been implicated in stress response and tolerance, and specifically in protection from osmotic stress (Kaino and Takagi, 2008). Glycerol metabolism genes have been implicated in hexane tolerance in the solvent-tolerant *E. coli* strain OST3410 which, compared to the parent strain, expresses higher levels of the glycerol metabolism genes *glpB*, *glpC* (glycerol-3-phosphate dehydrogenase subunits), *glpF* (glycerol uptake facilitator), and *glpQ* (glycerophosphoryl diester phosphodiesterase) (Hayashi et al., 2003). Higher expression of *glpC* has also been observed in other solvent-tolerant *E. coli* strains following treatment with xylene and cyclohexane (Shimizu et al., 2005). The involvement of altered amino acid and glycerol metabolism in the response of *C. acetobutylicum* to butanol stress has been implicated by recent microarray studies (Alsaker et al., 2010), but detailed studies are lacking to substantiate these implications.

In view of several reports to the effect that solvent (and some acid; see below) stress frequently involves an oxidative-stress component that results in damaged proteins, nucleic-acids, phospholipids and membranes, trehalose, proline and glycerol may be good protectants that remain to be explored in this context for solvent and possibly acid tolerance in microorganisms.

3. Carboxylic-acid stress & tolerance

In this discussion of carboxylic-acid stress and tolerance, we do not address classical acidophilic organisms (i.e., organisms that grow at very low pH, defined as pH less than about 2.0), but rather organisms that are likely to be exposed to such stress, either in their natural milieu or in a bioprocess. These would include, but not limited to *E. coli*, *Bacilli*, *Clostridia*, *Lactobacilli*, and yeast. The emphasis is on mechanisms to deal with stress from and tolerance to organic acids deriving from metabolic activities, rather than protons per se.

3.1. Which carboxylic acids and how are they transported across membranes?

Production of carboxylic acids is routine during growth of virtually all microorganisms, whether these acids are desirable or undesirable products of either or both aerobic and anaerobic cell metabolism (Papoutsakis and Meyer, 1985a, 1985b). These would include naturally produced acids such as acetic, lactic, butyric, citric, malic, fumaric, succinic, amino acids (e.g., aspartic and glutamic), propionic, and formic acids, but also several other which are produced at higher concentrations by engineered organisms (e.g., 3-hydroxypropionic, (Warnecke and Gill, 2005)) or are used as preservatives (e.g., sorbic or benzoic acids). In the context of bioprocessing for the production of chemicals, such as butyrate (Jones et al., 2008), succinate (Andersson et al., 2009) or 3-hydroxypropionate (Warnecke and Gill, 2005), development of acid-tolerant strains is essential for achieving higher cell densities, growth and sustained metabolism (Warnecke and Gill, 2005).

By producing certain carboxylic acids, such as acetate, butyrate or succinate, microorganisms generate ATP via substrate-level phosphorylation (Papoutsakis and Meyer, 1985a, 1985b). For fermentative bacteria, such as *C. acetobutylicum*, substrate-level phosphorylation leading to acid (butyric and acetic) production is the primary means of ATP generation. However, acid accumulation during active growth is toxic to cells (Papoutsakis et al., 1987; Russell, 1992). A well-known example is acetate accumulation

during *E. coli* growth, and especially so at high cell densities. In some cases, acids can be simultaneously growth inhibitory and solvent-formation stimulants. A well-known example is the production of butyrate and/or acetate by solventogenic, acetogenic and cellulolytic *Clostridia* during exponential growth. Although *Clostridia* are generally viewed as acid tolerant, accumulation of butyrate and/or acetate lowers the culture pH leading either to a genetic switch to solvent production in solventogenic *Clostridia* (e.g., *C. acetobutylicum* ATCC 824, *C. beijerinckii*, and *C. cellulolyticum*) (Paredes et al., 2005; Zhao et al., 2005) or cessation of metabolism in non-solventogenic *Clostridia* (e.g., *C. acetobutylicum* M5, *C. butyricum*, and *C. tyrobutyricum*). For these organisms, acid re-uptake that detoxifies the cytosol is only possible when solvents can be produced: butyrate is taken up and converted to butyryl-CoA, which is then converted to butanol. Acetate is taken up and converted to acetyl-CoA, which is then converted to butyryl-CoA or to ethanol. Similarly acetate, but not lactate, can stimulate ethanol formation in prokaryotic ethanologens (He et al., 2009); this is presumably because acetate uptake leads to acetyl-CoA formation which can then be converted to ethanol.

How do carboxylic acids get out or into cells? Many undissociated (i.e., protonated) organic acids may either diffuse freely across the cell membrane or may be assisted via facilitated transport by generic or specialized permeases/channels; both are ATP-independent processes. In either case, they may affect cellular physiology through both free proton and anion interactions (Kell et al., 1981; Papoutsakis et al., 1987; Russell, 1992; Russell and Diez-Gonzalez, 1998; Walter and Gutknecht, 1984; Warnecke and Gill, 2005). Furthermore, many cells, especially yeast cells, contain specialized and/or general active (i.e., energy dependent) transporters for carboxylic acids, which are of two types: molecular pumps to expel the acids from the cytosol or permeases for importing acids into cells (Casal et al., 2008). Active transporters are essential components of both acid uptake (such as in the case where the acids are carbon and/or energy substrates) but also acid detoxification by excretion. Bacteria may also express carboxylic-acid transporters, but generally those are restricted to transporters for the utilization or excretion of dicarboxylic acids, such as succinate, fumarate, malate, the dicarboxylic amino acid aspartate (Janausch et al., 2002) or for acetoacetate uptake/transport (AtoE, coded as part of the *ato* operon in *E. coli*) (Matta et al., 2007). Some of these transporters are engaged in the glutamate- and arginine-decarboxylation dependent mechanisms for acid resistance/tolerance which are discussed below.

3.2. How carboxylic-acids inhibit cells: membrane uncoupling, enzyme inhibition, DNA damage (and a broader oxidative-stress damage?)

The deleterious effects that result from accumulation of carboxylic acids in cells derive from several possible processes due to proton or anion accumulation (Russell, 1992). An important process that results in inhibition of growth and metabolism is membrane uncoupling (Baronofsky et al., 1984; Herrero et al., 1985; Huesemann and Papoutsakis, 1986; Kashket, 1987), which refers to reduction of the membrane protonmotive force (Δp) by reducing the transmembrane ΔpH (Russell, 1992; Russell and Diez-Gonzalez, 1998). Furthermore, accumulation of excess free protons may damage ribosomal RNA as well DNA (Raja et al., 1991; Sinha, 1986), and alter enzymatic activities in both general and specific fashion (Abbott et al., 2009; Beales, 2004; Serrazanetti et al., 2009). The effects of carboxylic acids is generally strongly pH-dependent, but non-pH-dependent

mechanisms also exist (Beales, 2004). Generally, however, growth inhibition is associated with the concentration of the undissociated carboxylic acids (Beales, 2004; Huesemann and Papoutsakis, 1986; Huesemann and Papoutsakis, 1988). Significantly, acid stress is now increasingly viewed as partially at least similar to oxidative stress (Abbott et al., 2009; Alsaker et al., 2010; Martin-Galiano et al., 2005). In yeast cells, acid-mediated generation of reactive-oxygen species (ROS) is attributed to two possible mechanisms (Abbott et al., 2009): in the first, acids increase the respiration rate, which leads to higher ROS levels; in the second, carboxylic acids may stimulate generation of hydroxyl radicals via the iron-ion catalyzed Fenton reaction. It is also possible that induction of apoptosis due to acid stress in yeast cells may be linked to oxidative-stress generated ROS species. Evidence for an oxidative-like acid-stress response in bacteria is discussed in the following sections.

3.3. The three established acid-resistance (AR) systems: *E. coli* and beyond

For general acidity in the form of excessive cytoplasmic protons or low pH (inside and outside the cell), *E. coli* engages mainly three acid-resistance (AR) systems, as reviewed in detail by Foster (2004). AR1 is not well understood other than it involves the alternate sigma factor σ^S (see Box 2), the global network of the cAMP receptor protein (CRP), the F_0/F_1 ATPase, and that it is glucose repressed. The other two systems involve the decarboxylation and antiport of the amino acids glutamate (AR2) and arginine (AR3); these are not ATP dependent, and contribute to acid tolerance mostly in Gram⁻ organisms such as *E. coli*, and species of *Shigella*, *Listeria*, but likely many more (Foster, 2004). Amino acid decarboxylation results in CO₂ production, proton consumption, and generation of either γ -amino butyric acid (GABA) or agmatine (by decarboxylation of glutamate and arginine, respectively) which are exchanged by an antiporter for glutamate or arginine, respectively (Foster, 2004; Richard and Foster, 2004). As a result, decarboxylation coupled with antiport of the decarboxylation products for additional amino acid generates a net efflux of cytoplasmic protons thus increasing the cytoplasmic pH.

3.4. Resistance to acetate stress using amino acids other than glutamate and arginine

In *E. coli*, acetate stress and inhibition can be mitigated by addition of the amino acids methionine, glycine, threonine, and isoleucine (Han et al., 1993; Roe et al., 2002). Also, acetate was shown to upregulate select amino acid transporters (Kirkpatrick et al., 2001), presumably in order to enhance the transport of select amino acids whose accumulation intracellularly offers protection against acetate stress. In *C. acetobutylicum*, the overall upregulation of methionine and select amino acid transporters in response to acetate stress is consistent with the *E. coli* findings and suggests a preservation of mechanisms whereby accumulation of certain amino acids can relieve acetate stress (Alsaker et al., 2010).

3.5. Membrane composition and acetic-acid tolerance

There is also experimental evidence that acid tolerance is impacted by the composition of the cell membrane (Hanada et al., 2001). Organisms of the *Acetobacter* and *Glucanobacter* genera, known as acetic acid bacteria, have high resistance to acetic acid. Genes related to their acid resistance were cloned by complementation using acetic acid sensitive mutants of *A. aceti*, but their

role remains elusive. Phosphatidylcholine (PC) is a major component of membranes in several bacteria, including those of the genus *Acetobacter*. Using a gene-inactivation strategy, it was shown that, in *A. acetii*, PC in the cell membrane is necessary for its high acetic-acid tolerance.

3.6. Genomic-level acid stress response: HSPs, energy, iron and riboflavin metabolism, and metal transporters

There is a growing literature on genomic-level studies, by microarray and proteomic analyses, to examine the impact of carboxylic-acid stress in both prokaryotic (Alsaker et al., 2010; Jakob et al., 2007; Kannan et al., 2008; Martin-Galiano et al., 2005; Pieterse et al., 2005; Polen et al., 2003; Serrazanetti et al., 2009) and yeast cells (Abbott et al., 2007, 2009; Kawahata et al., 2006). Several common sets of genes and programs were identified in most of these studies. They include HSP genes of the general stress response including *groESL*, *dnaK*, and genes related to energy metabolism. In addition to these general responses, there were several patterns conserved among various prokaryotes and yeast cells, and additional patterns more specific to various organisms. These are briefly discussed below.

Among prokaryotes, transcriptional and proteomic data in *Lactobacilli* (Serrazanetti et al., 2009) demonstrated induction of the stringent response system, engagement of the amino-acid decarboxylation systems (discussed above), strain-dependent stress adaptation of the protein-synthesis machinery, changes in the electron flow system, as well as induction of fatty-acid synthesis genes. In *L. plantarum* (Pieterse et al., 2005), the trehalose uptake and conversion system was also upregulated, suggesting that trehalose has a protective effect against lactic-acid stress as for the already discussed protection against solvent, osmotic and desiccation stress. In *Corynebacterium glutamicum*, microarray and gene-deletion analysis (Jakob et al., 2007) showed the importance of σ^B (its deletion reduces acid tolerance) and σ^E (its deletion improves acid tolerance, but otherwise retards growth) in acid tolerance; their microarray data showed upregulation of iron transporters and of genes important to DNA-damage repair (apparently to deal with low pH/high proton-concentration induced DNA damage (Raja et al., 1991)). Genes/programs affected by acid stress (and associated with lactic-acid production) in *Streptococcus pneumoniae* included upregulation of manganese and iron transporters, upregulation of genes involved in pyrimidine and riboflavin biosynthesis; their data show an extensive overlap of acid-stress genes with genes associated with oxidative- and osmotic-stress responses (Martin-Galiano et al., 2005). In an *E. coli* microarray study of the adaptive response to acetate and propionate stress (Polen et al., 2003), in addition to the general σ^S -controlled stress response, the response included upregulation of chemotaxis and flagellar genes, downregulation of sugar, amino acid and amino sugar genes, and, for propionate stress only, upregulation of the threonine and isoleucine biosynthesis genes. In another study involving pH stress with and without recovery of cellular metabolism (Kannan et al., 2008), in addition to genes associated with amino acid decarboxylation (see above), there was upregulation of genes of: glycerol transport, osmotic-stress response, of the Fur (ferric uptake regulator) regulon, oxidative stress response, succinate dehydrogenase, biofilm-associated genes, and members of the Gad and Rcs regulons. In addition to the general upregulation of most HSP genes, and changes in energy metabolism, in *C. acetobutylicum*, acetate and butyrate stress had a more complex impact, some common between the two acid stresses and some different or opposite (Alsaker et al., 2010). Motility genes were downregulated by acetate only, methionine biosynthesis and

nitrogen-fixation genes were upregulated by acetate only, and purine biosynthesis genes were upregulated by both acids. Butyrate stress upregulated many iron-metabolism genes, riboflavin-biosynthesis genes and many genes associated with cellular repair from oxidative damage (Alsaker et al., 2010). Thus, in prokaryotes at least, oxidative stress appears to be involved in or be part of the cellular response to carboxylic-acid stress. As noted previously, oxidative stress appears to be part of the cellular response to solvent stress, as well.

In *S. cerevisiae*, acetate and lactate stress were investigated by both microarray analysis and functional screening of strain collection of non-essential-gene deletions (Kawahata et al., 2006). Notable was the upregulation of genes of metal metabolism regulated by the transcription factor Aft1p. Both analyses suggested that acid stress affects cell wall biosynthesis. Microarray analysis of transcriptional responses to four weak organic acids (benzoate, sorbate, acetate and propionate) in anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* (Abbott et al., 2007) revealed that the strongly lipophilic acids (benzoate and sorbate) affected genes related to the cell wall biosynthesis, while acetate and propionate had a stronger impact on membrane-associated transport processes. Overall, this study found that *S. cerevisiae* exhibits a minimal common transcriptional response (14 genes) among the four weak organic acids examined. Another microarray analysis examined the lactic acid vs. the lactate stress by a changing pH and total acid concentrations in a chemostat (Abbott et al., 2008). The study concluded that iron homeostasis plays a major role in the response to high lactate concentrations, and that the regulon of the transcription-factor Haa1p regulates the response to high concentrations of undissociated lactic acid.

3.7. The SRP system: a rarity or a general “General”?

An emerging mechanism of acid tolerance has been investigated in the Gram⁺ *Streptococcus mutans* (Gutierrez et al., 1999; Kremer et al., 2001). It involves the signal recognition particle (SRP), which is a ribonucleotide-protein complex composed of a small cytoplasmic RNA (scrRNA) and Ffh, the fifty-four homologue (Ffh) protein, a GTPase. The bacterial SRP is the prokaryotic counterpart of the well-studied eukaryotic SRP system, which is responsible for the cotranslational membrane targeting of signal-peptide-bearing secretory and membrane proteins to the plasma membrane. SRP functions by direct interaction with the ribosome: both the scrRNA and Ffh recognize and bind to both protein and nucleic-acid components of the ribosome, although the precise mechanism of this apparently complex interaction is not well known (Gu et al., 2005; Rinke-Appel et al., 2002). SRP recognizes which proteins are membrane or secreted proteins and moves their biosynthesis to the membrane by virtue of recognition of the Ffh receptor, FtsY. Loss of Ffh activity results in loss of acid tolerance (Kremer et al., 2001), but the mechanism for this lost tolerance is not known. One hypothesis might be that in the absence of Ffh and thus SRP function, membrane proteins cannot be accurately targeted to the membrane and thus membrane processes and functions will suffer. This could affect the function of membrane transporters, which as discussed above, play a major role in acid (but also in solvent) tolerance in most microorganisms. In this respect, loss of the SRP “General”, which orchestrates membrane targeting of proteins, might impact all membrane processes and not only those associated with acid tolerance. This remains to be tested. Some evidence for the importance of the SRP system in other organisms has been recently provided by our group (Borden et al., 2010). Using a sheared-DNA genomic library of *C. acetobutylicum*, whereby inserts can be expressed in both directions from a strong promoter, serial transfer of

library-bearing *C. acetobutylicum* cells exposed to increasing butyrate concentrations enriched for inserts containing fragments of ribosomal RNA (rRNA) genetic loci. It was determined that the selected library inserts were placed so that antisense (to the rRNAs) non-coding RNAs (ncRNAs) were transcribed. A minimal tolerance fragment was identified as the 16S-rRNA promoter region. Expressed from a plasmid, this tolerance fragment imparted superior resistance to butyrate and other carboxylic acids. Transcriptional analysis of butyrate stress identified 120 differentially expressed genes between the fragment-overexpressing and the control strains. The few upregulated genes included the *fffh* gene of the SRP system.

4. Inhibitors in hydrolyzed lignocellulosic feedstocks

Hydrolysis of lignocellulosic material is necessary to generate an accessible feedstock for subsequent fermentation. A drawback of this hydrolysis treatment is the generation of compounds which inhibit cell growth and product formation, thus reducing the fermentability of the hydrolyzed feedstocks, and therefore the productivity of the process. In order to mitigate this problem, several hydrolysis methods involving pretreatment, cellulose breakdown, conditioning, as well as detoxification have been developed, but none to the point where biomass hydrolysates are free of inhibitors. Thus, strain development to generate more tolerant strains would be beneficial for process development to increase the productivity of lignocellulosic-based fermentations. Several reviews have been recently published on the issue of inhibitors present in lignocellulosic hydrolysates (Almeida et al., 2007; Mills et al., 2009; Palmqvist and Hahn-Hagerdal, 2000; Pienkos and Zhang, 2009), and therefore only a brief summary is presented here. The aim is to connect the findings regarding these inhibitors to those for solvents and carboxylic acids already discussed.

In order to ferment lignocellulosic material, the key components, cellulose and hemicelluloses (xylan), must be decomposed to their monomeric or oligomeric building blocks, and also be separated from the non-fermentable lignin component. Usually this is performed in a two-step process involving a pretreatment to degrade hemicellulose and remove lignin followed by cellulose hydrolysis. Hydrolysis can be achieved chemically (dilute-acid hydrolysis) or enzymatically (cellulases). Enzymatic hydrolysis can also be carried out simultaneously with fermentation, a process termed SSF (simultaneous saccharification and fermentation) (Philippidis et al., 1993). Pretreatment methods can be classified as mechanical, chemical and enzymatic, and combinations thereof (Pienkos and Zhang, 2009; Sun and Cheng, 2002). Depending on the pretreatment method used, several inhibitory compounds can be generated during hydrolysis of lignocellulosic feedstocks. These inhibitory compounds can be classified into three major groups: (i) furan derivatives, with 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) as the most abundant compounds; (ii) weak carboxylic acids, mainly acetic, formic and levulinic acid; and (iii) phenolic compounds, such as vanillin and syringaldehyde from the degradation of lignin.

The furan derivatives HMF and furfural are formed at high temperatures and pressure as degradation products of hexoses and pentoses, respectively (Palmqvist and Hahn-Hagerdal, 2000). The concentrations of these chemicals vary depending on the source of biomass feedstock as well as pretreatment method; the concentration of HMF is usually higher than that of furfural (Almeida et al., 2007). Weak acids are formed through deacetylation of hemicellulose (acetic acid) or through the further degradation of HMF and furfural (formic and levulinic acids) (Palmqvist and Hahn-Hagerdal, 2000). Phenolic compounds have their origin in the degradation of lignin and of carbohydrates.

Lignin content and composition differs between lignocellulosic feedstocks (hardwood, softwood or herbaceous material). Thus, there is large variation in the composition as well as the amounts of phenols generated during the pretreatment process (Klinke et al., 2004). Table 1 summarizes what is broadly known regarding mechanisms by which these toxic compounds inhibit cell growth and product formation. In general, furan derivatives inhibit energy metabolism through inhibition of key enzymes (Modig et al., 2002) as well as through depletion of cofactor pools. One of the major defense mechanisms in yeast cells is the reduction of furfural and HMF to their less toxic alcoholic forms using NAD(P)H dependent reductases like alcohol dehydrogenase ADH6 (Larroy et al., 2002). The weak acids also influence the energy metabolism of the cell by disrupting the membrane electrochemical potential used to generate ATP (Palmqvist and Hahn-Hagerdal, 2000). Phenols in biomass hydrolysates constitute a very diverse group and, so far, a general mechanism of inhibition has not emerged, but it is believed that they mainly act on biological membranes and lead to a loss of membrane integrity (Heipieper et al., 1994).

To overcome the negative effects of these inhibitors, recent research attempts have focused on the improvement of the fermentation organism, whereby the strategies range from classical strain development to systems-biology-based approaches. In classical strain development, organisms were exposed to increasing concentrations of the inhibitor or directly to inhibitor-containing hydrolysates leading to the selection of strains with improved tolerance (Liu et al., 2005; Martin et al., 2007). A similar strategy was used in an evolutionary-engineering approach, whereby an iterative process of genetic diversification followed by selection was applied to find mutants with increased tolerance. Such a strategy, utilizing UV-mutagenesis, has led to the identification of a tolerant mutant of the pentose-fermenting yeast *Pichia stipitis* (Bajwa et al., 2009). Rational methods have focused on developing tolerant strains towards furfural by over expressing NAD(P)H depending oxidoreductases like alcohol dehydrogenase ADH6, which reduces furans to their less toxic alcoholic forms (Larroy et al., 2002; Petersson et al., 2006). In another approach, the enzyme laccase from *Trametes versicolor* was expressed in *S. cerevisiae* and was shown to result in enhanced tolerance towards phenolic inhibitors (Larsson et al., 2001a). Laccase is a *p*-diphenol oxidase that catalyzes the reduction of molecular oxygen to water and the one-electron oxidation of phenolic compounds to radicals, which are unstable and eventually form high-molecular-mass non-toxic polymers. In another study (Larsson et al., 2001b), the gene *pad1p* coding for a phenylacrylic acid decarboxylase was overexpressed in *S. cerevisiae*. Pad1p catalyses a decarboxylation step by which aromatic carboxylic acids are converted to the corresponding non-toxic vinyl derivatives. The genetically modified strain could not only convert the model aromatic carboxylic acids ferulic and cinnamic acids faster than the parental strain, but also showed superior growth and ethanol production characteristics when grown on dilute acid hydrolysates of spruce.

A reported genomic-based approach employed a gene disruption library of *S. cerevisiae*. The library was exposed to furfural which led to the identification of several pentose phosphate pathway (PPP) genes in growth-deficient mutants (Gorsich et al., 2006). Further studies revealed that the gene *zwf1*, coding for a glucose-6-phosphate dehydrogenase, increased the tolerance towards furfural (Gorsich et al., 2006). Another genomics-based report aimed to increase the tolerance of an ethanologenic yeast *Candida krusei* towards acetic acid. Several rounds of whole genome shuffling (see below) with genetic diversification through UV-mutagenesis led to the isolation of a mutant with increased acetic acid tolerance as well as increased tolerance towards ethanol, freeze-thaw, H₂O₂, and heat shock (Wei et al., 2008).

Table 1

Overview of inhibitors present in hydrolyzed lignocellulosic material, their effect on fermentation as well as their presumed mechanism of inhibition.

Compound	Origin	Effect on fermentation and observed characteristics	Proposed or assumed mechanism of inhibition
Furans			
Furan-2-carbaldehyde (furfural)	Degradation of pentoses	<ul style="list-style-type: none"> growth is generally more inhibited than ethanol production (Palmqvist et al. 1999a) ethanol yield and production rate are reduced mainly because of reduced biocatalyst amount 	<ul style="list-style-type: none"> inhibits energy metabolism (inhibition of the enzymes alcohol and pyruvate as well as aldehyde dehydrogenase in vitro (Modig et al., 2002), and hexokinase as well as glyceraldehyde-3-phosphate dehydrogenase in crude extracts (Banerjee et al., 1981))
5-hydroxymethyl-2-furaldehyde (HMF)	Degradation of hexoses	<ul style="list-style-type: none"> leads to an increased lag phase in <i>S. cerevisiae</i>, which metabolizes furfural and HMF to their less toxic alcoholic forms (Palmqvist et al. 1999a) furfural is also metabolized by <i>E. coli</i> LY01 (Zaldivar et al. 1999) furfural is more toxic than HMF and synergistic effects between them have been observed (Almeida et al., 2007) 	<ul style="list-style-type: none"> depletion of NAD(P)H through reduction of furfural and HMF to their alcoholic forms (Palmqvist et al. 1999a) furfural inactivates cell replication (Palmqvist et al. 1999a)
Weak acids			
Acetic acid	Hemicellulose degradation	<ul style="list-style-type: none"> primarily inhibits growth of the biocatalyst (<i>E. coli</i> LY01 (Zaldivar and Ingram 1999)) and ethanol yield (<i>S. cerevisiae</i> (Larsson et al. 1999)) 	<ul style="list-style-type: none"> two mechanisms are proposed to explain inhibitory effects (Russell 1992)
Formic acid	furfural and HMF degradation	<ul style="list-style-type: none"> low concentrations stimulate ethanol production (Larsson et al. 1999; Zaldivar and Ingram 1999) 	<ul style="list-style-type: none"> uncoupling of the membrane proton-motive force intracellular-pH drop caused by inflow of undissociated acid is compensated through active transport of H⁺ out of the cell at the expense of ATP
Levulinic acid	HMF degradation	<ul style="list-style-type: none"> no synergistic effects of the three listed acids was observed (Larsson et al. 1999; Zaldivar and Ingram 1999), whereby synergistic effects of acetic acids and furfural was observed for <i>E. coli</i> LY01 (Zaldivar and Ingram 1999) and <i>S. cerevisiae</i> (Palmqvist et al. 1999b) toxicity of the weak acids is associated with their hydrophobicity (high hydrophobicity—more toxic) (Larsson et al. 1999; Zaldivar and Ingram 1999) 	<ul style="list-style-type: none"> Intracellular anion accumulation carboxylic acids accumulate in the cell as their anionic form leading to further inflow of undissociated acids in order to equilibrate the intracellular and extracellular spaces, thus resulting in high anion accumulation both mechanisms result eventually in the collapse of the membrane ΔpH, thus causing the breakdown of the electrochemical membrane potential needed for energy generation
Phenolic compounds			
4-hydroxybenzaldehyde/-acid Vanillin/vanillic acid Syringaldehyde/syringic acid	Generally from lignin breakdown (solubilization, hydrolytic or oxidative cleavage of lignin)	<ul style="list-style-type: none"> biomass yield, growth rate and ethanol productivities are reduced (Almeida et al., 2007) very diverse group including aldehydes, ketones, acids and others low molecular weight (MW) phenols are more toxic than high MW (Buchert et al., 1989) also the substituent position on the aromatic ring influences the toxicity (Klinke et al., 2004) for an overview of aromatic compounds present in lignocellulosic hydrolysates see (Klinke et al., 2004) 	<ul style="list-style-type: none"> because of the diversity of this group no generalized mechanism was elucidated so far it is assumed that phenols partition into membranes and therefore effect the membrane integrity (Heipieper et al. 1994) toxic effects can be reduced through the lignin oxidizing enzyme laccase (Jonsson et al. 1998)

5. Tolerance for and lessons from bioremediation

Bioremediation employs both naturally selected as well as genetically engineered strains to remediate simple or complex mixtures of pollutants (Diaz, 2004; Pieper and Reineke, 2000; Symons and Bruce, 2006). While the majority of applications so far have focused on using naturally selected strains or consortia, there is increasing interest in engineering strains (Haro and de Lorenzo, 2001; Pieper and Reineke, 2000) with more complex capabilities. Such strains are meant to both tolerate and degrade one or mixtures of toxic chemicals (Haro and de Lorenzo, 2001; Pieper and Reineke, 2000). Most of such efforts have focused on developing pathways and reaction capabilities for detoxification of xenobiotics, and hardly any effort specifically to enhance tolerance to chemicals as a means to facilitate faster metabolism, and thus detoxification rates, of the targeted chemicals. Nevertheless, this remains an important need, and thus, endowing organisms with tolerance to solvents or other chemicals is highly desirable for bioremediation purposes (Pieper and Reineke, 2000).

Organisms that have been used or have been explored for use in bioremediation include some of the most solvent and broadly chemical tolerant species known. Most of that exceptional

tolerance remains little understood and even less explored in applications outside bioremediation. In addition to *Pseudomonas*, they include species of *Rhodococcus*, *Burkholderia*, *Enterobacter*, *Deinococcus*, *Bacillus*, and *Nocardioidea* (Pieper and Reineke, 2000). A few recent examples of the extraordinary tolerance of some of these organisms include the *Rhodococcus* sp. Moj-3449 which grows on 1–180 g/l n-hexane concentrations (Binazadeh et al., 2009), and *Deinococcus geothermalis* T27, which tolerates high concentrations of several solvents (having log P values ranging from 5.6 to as low as 0.7) provided as non-aqueous layers (up to 20% v/v) below or above the cell suspension (Kongpol et al., 2008). Other recent examples include species of the aforementioned genera that could tolerate up to 20% (v/v) of benzene, toluene or xylene (Wang et al., 2008); and *Rhodococcus erythropolis* DCL14 that can tolerate up to 52% (v/v) toluene (de Carvalho et al., 2007). There exist also reports on yeast species that can tolerate very high concentrations of diesel oil (30 g/l), phenol (2 g/l) and formaldehyde (7.5 g/l) (Kaszycki et al., 2006). These and many other examples demonstrate the enormous natural tolerance diversity that remains to be explored for the development of tolerant organisms for all bioprocessing applications.

6. Stress response vs. tolerance

6.1. Is the response to chemical stress general or specific? Adaptive or random? Beneficial or inconsequential?

As already discussed, toxic chemicals may elicit a variety of cellular responses whose nature may depend on evolutionary adaptation or general responses triggered by messages deriving from damaged macromolecules or cellular components. For chemicals that a cell has routinely encountered in its physiological milieu, programmed responses would be expected as part of the cell's evolutionary adaptation, and such responses would likely provide tolerance and protection to the cells. A recent genomic-level analysis (Alsaker et al., 2010) of such a response is that of the anaerobe *C. acetobutylicum* responding to butanol, butyrate or acetate, which are naturally produced by this organism. If however a cell has not encountered a specific toxic chemical before, the response is likely to derive from general mechanisms sensing protein or nucleic-acid damage or unfolding, production of small molecules or radicals, such as reactive-oxygen species (ROS), malfunctioning cellular programs such as membrane processes and related energy generation processes, or the impact of the solvent or chemical on the biophysical processes that orchestrate core cellular programs. The latter could include protein–DNA binding, protein–protein interactions, protein embedding in membranes, nucleic-acid interactions, etc. Two genome-scale studies have been recently reported (Brynildsen and Liao, 2009; Rutherford et al., 2010) describing how *E. coli* responds when exposed to n- and i-butanol, two chemicals unlikely to have been encountered by this organism. Perturbation of respiration and oxidative-like stress responses were key characteristics of these responses. This could be contrasted to the butanol-stress responses of *C. acetobutylicum* (Alsaker et al., 2010), which produces butanol. These responses are limited to

upregulation of genes of the general HSP/chaperone system (to protect the cellular machinery), genes of the primary metabolic pathways, and differential expression of genes belonging to specialized programs such as fatty-acid biosynthesis, motility and chemotaxis.

The issue of whether a cellular response to chemical toxicity may immediately or eventually lead to counteracting the toxic effect or the development of tolerance has been debated and remains largely unresolved. Logically, one would expect that some of the stress responses would immediately or eventually counteract the toxic insult, but many others may not. The reasoning is that, as suggested above, while some toxic chemicals may elicit evolutionarily programmed defense responses, many others do not necessarily engage specific programs and, thus, such responses may not result to protection. Even for chemicals that likely engage evolutionarily adapted responses (e.g., yeast responses to ethanol), the solvent, at higher concentrations, may elicit secondary responses deriving from signals of protein, lipid or nucleic-acid damage, and such responses may not offer any protection benefit.

6.2. What is the evidence that stress response is related to tolerance? Can we use stress response to enhance tolerance? What type of genomic studies would provide the best leads?

While there is not a definitive answer to these questions, a review of the literature may provide some limited perspective. Table 2 lists a select set of references of successful metabolic engineering efforts to generate tolerant strains, and includes some genome-scale investigations to understand and/or generate tolerant phenotypes. When considering the most general stress response, namely the upregulation of HSP proteins, it is evident (discussed in the HSP section above) that the upregulation of some of HSP proteins individually (e.g., GroES, GroEL and select

Table 2

A selection of references on the development of tolerant phenotypes using metabolic engineering, including genomic and global engineering approaches.

Strain	Solvent/stress	Approach	Result	Reference
Wild-type <i>Lactobacillus</i>	Acid stress	Whole genome shuffling (WGS). Wild type (WT) cultures adapted in chemostats or mutated with nitrosoguanidine (NTG) and protoplast fusions were utilized for WGS	Increased acid tolerance to facilitate growth at pH 3.8 compared to pH 4.4 of WT. Three-fold increase in production of lactic acid	(Patnaik et al., 2002)
<i>Lactobacillus plantarum</i>	Heat stress, cold stress and solvent tolerance	Over-expression of three small heat shock proteins (sHSP)	Enhanced thermotolerance, improved resistance to cold temperatures, and increased ethanol and butanol tolerance	(Fiocco et al., 2007)
<i>Clostridium acetobutylicum</i> ATCC 824	Butanol	Screening of plasmid-based genomic DNA libraries and microarray analysis	Increase of 13% and 81% butanol tolerance based on batch culture or serial enrichment	(Borden and Papoutsakis, 2007)
<i>Clostridium acetobutylicum</i> ATCC 824	Butanol	Overexpression of <i>groESL</i> in a plasmid under the thiolase promoter	Decreased inhibition by butanol and increased solvent titers produced. Prolonged metabolism under butanol stress	(Tomas et al., 2003)
<i>Escherichia coli</i>	Heat stress	Artificial transcription factor libraries of zinc finger-containing motifs	The down-regulation of <i>ubiX</i> allows for thermotolerance	(Park et al., 2005)
<i>Escherichia coli</i>	Ethanol stress. Simultaneous ethanol and sodium dodecyl sulfate (SDS) stress.	Global transcription machinery engineering (gTME) of a sigma factor ($\sigma 70$)	Ethanol tolerance up to 60 g/L, simultaneous tolerance to ethanol and SDS, as well as lycopene overproduction	(Alper and Stephanopoulos, 2007)
<i>Saccharomyces cerevisiae</i>	Ethanol, 1-propanol and 1-pentanol	Deletion library screening	Identification of 137, 122, and 48 genes important in ethanol, 1-propanol, and 1-pentanol tolerance	(Fujita et al., 2006)
<i>Saccharomyces cerevisiae</i>	Furfural	Deletion library screening	Four genes (<i>zwf1</i> , <i>gnd1</i> , <i>rpe1</i> , and <i>tkl1</i>) in the pentose phosphate pathway increase tolerance to furfural. <i>Zwf1</i> over-expression allowed for growth in toxic furfural levels	(Gorsich et al., 2006)
<i>Saccharomyces cerevisiae</i>	Ethanol	Global transcription machinery engineering (gTME). Transcription factor Spt15p used in mutant libraries for ethanol production and tolerance	Enhanced viability of cells in 15% ethanol by volume. Increased efficiency in glucose conversion to ethanol	(Alper et al., 2006)

small HSPs) provides tolerance to solvent and possibly acid stress. Optimized expression of these proteins, let alone in a suitable ensemble, would be expected to provide even better tolerance, given the exquisite regulation and team action of the HSP response. If one examines the general stress response of solvents, namely their impact on membrane fluidity and functions, virtually all known cellular responses that counteract these effects have been accompanied by enhanced tolerance, yet no engineered organisms have yet been developed based on that principle. Also, the upregulation of efflux pump genes upon solvent stress observed in some microarray or other genomic-scale studies is consistent with the findings that several of these genes provide tolerance upon upregulation in engineered strains as already discussed. Similarly for the rather general upregulation of biosynthesis of osmolites like trehalose, whose accumulation appears to provide protection against both solvent and acid stress as already discussed. While not every single differentially expressed gene would be expected to provide tolerance upon engineered expression, much of such information may provide useful clues as to of desirable targets for strain engineering. If genomic scale studies had focused on cellular programs and pathways and their possible physiological connection to tolerance, creative solutions to engineer such programs, such as by engineering transcription or sigma factors affecting these programs, would be more likely to provide positive outcomes. Three recent systems-level studies, two on the impact of the unnatural (for *E. coli*) stressants butanol and i-butanol on *E. coli* (Brynildsen and Liao, 2009; Rutherford et al., 2010), and one a comparative analysis of naturally produced stressants (butanol, butyrate, and acetate) (Alsaker et al., 2010), generate several such leads for future strain development.

There is also an element of appropriate design in genome-scale studies (transcriptomic or proteomic) examining the stress response to a toxic chemical. The grand majority of such studies was not intending for the results to be used in generating tolerant phenotypes. One could logically argue that if a genome-scale study is to identify genes and programs which, upon up- or down-regulation, would confer chemical resistance, then such studies ought to be done on cells that have been adapted to some increased level of tolerance. A scheme for such studies is summarized in Fig. 2. Thus, up- or down-regulated genes and programs would be more directly associated with the tolerant phenotype. Yet, most studies have been carried out to examine the immediate stress response, rather than the adaptive changes that have led to the tolerant phenotype. Two notable exceptions have already been discussed, namely the microarray analyses of the adaptive response of *E. coli* to acetate and propionate stress (Polen et al., 2003), and the transcriptional analysis of long-term adaptation to high lactate concentrations of *C. glutamicum* (Jakob et al., 2007). These studies, combined perhaps with computational-analysis-based programs and pathway analysis could serve as paradigms of how one may identify programs and pathways for engineering tolerant strains.

7. Overlapping stress and tolerance among chemical stressants

Frequently, two or more chemical stressants co-exist in a fermentation broth such as for example ethanol with acetate or butanol with butyrate and acetate. Similarly, in most bioremediation applications, complex chemical mixtures must be tolerated and degraded to non-toxic chemicals. Thus, to analyze and understand such systems and provide strategies for developing tolerant strains against multiple co-existing stressants, one needs to have information for each stressant, plus, ideally some data

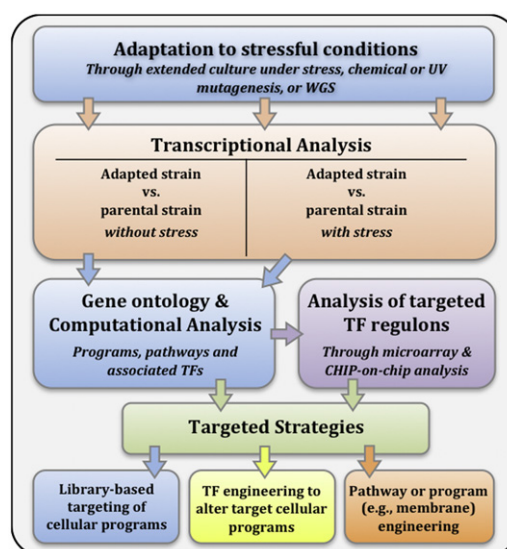


Fig. 2. Strategies for the identification of target genes and UVs, as well as for altering cellular programs to enhance chemical tolerance. After genetic diversification through mutagenesis or WGS (whole genome shuffling), and cultivation under stressful conditions to adapt strains to higher levels of chemical stress, strains are subjected to whole genome analysis, such as transcriptional and/or proteomic analysis. Comparison of the transcriptome (and/or proteome) of the adapted and parental strain under various environmental conditions should lead to the identification of altered cellular programs, pathways or genes beneficial to sustained tolerance against the applied stress. Identified transcription factors (TFs) can be subjected to further investigation by microarray or ChIP-on-chip analysis (Chromatin Immunoprecipitation analyzed by microarrays (chip)) to explore or elucidate their regulon. The generated information can then be used to develop strategies to target cellular programs, pathways or transcription factor (TF) regulons in order to engineer strains with improved tolerance characteristics. Such strategies can involve library approaches, TF engineering or the development of altered, semi-synthetic or synthetic cellular programs, such as novel membranes or stress-response systems.

from cultures where all stressants are present at realistic concentrations. There are no reported studies of the latter kind, except for an effort to identify programs affected by dramatically different product concentrations of solvents and carboxylic acids in recombinant strains of *C. acetobutylicum* (Tummala et al., 2003). When re-assessed in the context of a more recent study of individual responses to each chemical (butanol, acetate, butyrate) (Alsaker et al., 2010), the findings from that study suggest a largely composite response from the various chemicals (Tummala et al., 2003).

How much overlap is there among stress responses to different chemicals or tolerance towards different chemicals? We have discussed that most chemicals elicit the general HSP response, perhaps with some specialized differences in terms of the intensity of upregulation or the engagement of additional specialized stress proteins. This is shown systematically in a recent study on the response of *C. acetobutylicum* to butyrate, acetate and butanol (Alsaker et al., 2010). However, each chemical had its own distinct stress regulon, with some or significant binary overlap, e.g., some common programs between the butyrate and acetate stress response, etc. Thus, engineering strategies based on overexpression of the HSP system are likely to provide resistance to a broader spectrum of chemicals, but each chemical class and each specific chemical may also elicit a specialized response and may thus require more targeted strain engineering for tolerance. As already discussed, in a microarray study on the response of yeast cells to four carboxylic acids (acetate, propionate, sorbate and benzoate), a minimal common set of genes (14) was identified (Abbott et al., 2007). Significantly, however, if the responses are examined based on acid lipophilicity

(the moderately lipophilic benzoate and sorbate vs. the less lipophilic acetate and propionate), the common set of genes is much larger. Much remains to be studied, but these limited set of data would suggest engineering strategies that can generate general and specialized chemical tolerance at the same time, especially as tools are being developed to generate enlarged or semi-synthetic genomes (see below).

8. Genome-scale approaches for developing tolerant phenotypes

In addition to the reviewed traditional metabolic engineering approaches involving the overexpression or knockout of one or few genes, genomic or global approaches are proving increasingly successful in the development of tolerant phenotypes, and much of that work has been recently reviewed (Papoutsakis, 2008; Patnaik, 2008; Santos and Stephanopoulos, 2008; Warner et al., 2009). Thus, only a brief summary of these important developments with emphasis on the development of tolerant phenotypes is presented here. Table 2 lists a select set of references on genome-scale investigations or metabolic engineering to understand and/or generate tolerant phenotypes.

8.1. Whole genome shuffling (WGS)

In WGS, the DNA of similar but diversified cell populations is shuffled to recombine desirable genes (or mutations) in order to generate improved bacterial phenotypes. Sequence diversity prior to recombination comes from mutations of the parent organism generated by a mutagenic process. These mutations are then recombined (based on regions of homology) by repeated protoplast fusions. Mutational diversity prior to WGS can be achieved by chemostat-mediated adaptation (Patnaik et al., 2002), chemical mutagenesis (e.g., via nitrosoguanidine (NTG) treatment (Patnaik et al., 2002)) or ultraviolet (UV) radiation (Wang et al., 2007). Microbial populations generated by WGS can be then screened for tolerance to toxic chemicals and harsh fermentation conditions or for improved metabolite productivity (Dai and Copley, 2004; Patnaik et al., 2002; Wang et al., 2007; Zhang et al., 2002). WGS was successfully used with *Lactobacilli* (Patnaik et al., 2002; Zhang et al., 2002), but so far no reports emerged for *E. coli*, probably due to the difficulty of creating true protoplasts for fusing. In *Sphingobium chlorophenolicum*, WGS enabled improved degradation and higher tolerance to pesticide pentachlorophenol, which demonstrates both the generation of an improved phenotype and its application in bioremediation (Dai and Copley, 2004). Recently, WGS was successfully utilized in *Clostridia* for the production of 1,3-propanediol (1,3-PDO) in *C. diolis* DSM 15410 (Otte et al., 2009), where improved tolerance to glycerol and 1,3-PDO and an 80% yield improvement compared to the parent strain were demonstrated. Shi et al. (2009) successfully utilized genome shuffling in *S. cerevisiae* strain SM-3 to enhance thermotolerance, ethanol productivity and ethanol tolerance. Protoplasts were exposed to ultraviolet radiation, and after three rounds of protoplasts a yeast population that grew in temperatures as high as 55 °C and produced high ethanol yields was isolated (Shi et al., 2009). Furthermore, the strains exhibited tolerance up to 25% v/v ethanol, with viability up to 60% compared to the parent strain.

8.2. Transcription and sigma-factor libraries

Although transcription-factor libraries, and in particular zinc-finger transcription factors, generated by random mutagenesis or other techniques that generate sequence diversity, have been

extensively used in basic biological research since the early 1990s (Beerli and Barbas, 2002; Jamieson et al., 1994), use of such libraries was not explored for developing complex phenotypes until the early 2000s. Synthetic transcription factors containing zinc finger motifs from randomized libraries were screened for drug resistance, thermotolerance and osmotolerance in yeast and/or *E. coli* cells (Park et al., 2005, 2003). Thus, the entire transcriptome can be altered for phenotypic improvement by global transcription machinery engineering (gTME), whereby core transcription factors are mutated via error-prone PCR to generate diversity (Alper et al., 2006; Alper and Stephanopoulos, 2007). As reviewed (Santos and Stephanopoulos, 2008), transcriptional and thus phenotypic diversity can be also achieved by utilizing libraries of natural, semi-synthetic or artificial transcription factors. Most recently, this approach was used to select for mutants of the core sigma factor (coded by *rpoD*) of *L. plantarum* to select for strains tolerant to lactic and inorganic acids (Klein-Marcuschamer and Stephanopoulos, 2008).

8.3. Deletion libraries

Deletion libraries, which are constructed by disruptive mutations of many or most individual genes of a genome (only non-lethal disruptions are included in such libraries), can be screened under selective pressure to identify genes involved in tolerance to particular stressors. Disruptions of beneficial genes can be identified by changes in growth rate or survival on a selective medium, and those genes can be studied further to determine their role in tolerance. This availability of commercial deletion libraries for eukaryotes such as *S. cerevisiae* (Giaever et al., 2002) and prokaryotes like *E. coli* (Baba et al., 2006) has made screening a rather simple process. Furthermore, the availability of a bar-coded (using unique 20-mer DNA bar codes) deletion library for *S. cerevisiae* has expedited screening outcomes (Giaever et al., 2002). Fujita et al. (2006) utilized a homozygous diploid deletion library in *S. cerevisiae* and identified 137, 122, and 48 genes involved ethanol, 1-propanol, and 1-pentanol sensitivity. Other researchers have performed similar studies focusing specifically on ethanol (van Voorst et al., 2006). A similar deletion-library approach was used by Gorsich et al. (2006) to identify *S. cerevisiae* genes imparting tolerance to furfural, which, as discussed, is a product of cellulose degradation. A total of 62 genes were identified, and the authors focused on four genes, namely *zwf1*, *gnd1*, *rpe1* and *tkl1*, encoding for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, D-ribulose-5-phosphate-3-epimerase and transketolase-1, respectively. These genes have an active role in the pentose phosphate pathway, and were shown to influence furfural tolerance. These genes were individually overexpressed and it was concluded that *zwf1* increases tolerance and allows growth in the lethal concentration of 50 mM furfural (Gorsich et al., 2006).

Deletion libraries are a powerful tool for identification of important loci, but have some limitations. First, since they are single gene disruptions these libraries do not allow for the study of gene interactions in relation to the development of tolerance or any other complex phenotype. To remedy this limitation, the original *S. cerevisiae* library was used for the construction of double mutants and double knockouts are also being constructed in *E. coli* (Butland et al., 2008; Typas et al., 2008; Warner et al., 2009). Second, all knockouts need to be tagged for identification so genes can be tracked throughout the screening. Lack of such gene barcodes in the Keio collection can make the screening more challenging (Warner et al., 2009). This situation is likely to change; however, as a first report on the use of a bar-coded small *E. coli* library has just appeared (Hobbs et al., 2010). This library

was used to identify small non-coding RNAs involved in resistance to cell-envelope stress (see Box 2) and acid stress in *E. coli* (Hobbs et al., 2010). Finally, these libraries may be somewhat limited by the number of successful non-lethal disruptions present in the library.

8.4. Genomic libraries

Genomic libraries comprised of genomic DNA fragments on vectors provide a high or tunable copy number of genes and have been screened to identify desirable traits. There are genomic DNA, metagenomic, and cDNA libraries, and depending on the application, libraries can be constructed so that genes are expressed from a constitutive promoter, conditionally (from an inducible promoter), or using the gene's natural promoter(s).

8.4.1. cDNA libraries

Complementary DNA (cDNA) libraries were the first type of libraries to be used for screening for desirable genes (Liu et al., 1992) for functional-genomic applications of yeast and other eukaryotic cells. They can be constructed from mRNA isolated from a population via reverse transcription and screened for a particular function. cDNA libraries only contain the functional, coding elements of a genome, and can be placed under the influence of a promoter optimized for the host used. Their construction utilizes the poly-A tail to isolate mRNAs employing affinity columns. Originally used with an inducible promoter to identify genes whose overexpression is lethal or inhibitory to cells (Liu et al., 1992), their use was expanded to identifying genes that rescue known mutations or phenotypic defects, such as novel antioxidant genes (Lee et al., 1999). Due to lack of poly-A tails on mRNAs, bacterial cDNA libraries have not been extensively explored because it is not easy to separate mRNA from tRNA and rRNA. Nonetheless, it is possible to generate a library from the total RNA of a bacterial microorganism grown under stress.

8.4.2. Genomic-DNA libraries

Genomic-DNA libraries consist of digested or sheared DNA that is cloned into vectors, and transformed into cells that can then be screened for a selectable characteristic (Akada et al., 1997; Borden et al., 2010; Borden and Papoutsakis, 2007; Gill et al., 2002). They can be expressed from a strong constitutive promoter (Borden et al., 2010), or an inducible promoter (Akada et al., 1997) or the natural promoter of each gene/operon (Borden and Papoutsakis, 2007). The host carrying the library is exposed to some selective (e.g., faster growth rate) or stressful pressure (increasing concentrations of a toxic chemical) assuming that some gene(s) represented in the library will allow for favorable growth under such stress. This high-throughput method can be coupled with DNA microarrays to identify and isolate the enriched genes (Borden and Papoutsakis, 2007; Cho et al., 1998; Gill et al., 2002). Genomic library screens can thus be used to identify genes that improve a desirable phenotype or generate a novel phenotype. The method of screening of such genomic libraries can influence gene enrichment as demonstrated by Borden and Papoutsakis (2007). A genomic library of *C. acetobutylicum* DNA was constructed and screened in butanol-containing media. Serial transfers to media containing progressively higher butanol concentrations enriched for several genes, including four transcriptional regulators (CAC0977, CAC1463, CAC1869, and CAC2495). Over-expression of CAC1869 yielded an 81% improvement in butanol tolerance (Borden and Papoutsakis, 2007).

Genomic libraries have been used to quantitatively identify genes that lead to phenotypic improvement using a method termed SCAr Analysis of Library Enrichments (SCALEs) (Lynch

et al., 2007). This approach utilizes genomic libraries of different insert sizes that are individually transformed into the host cells. The combined transformants are used for selection, and plasmids contributing to an improved trait are enriched. Isolated DNA is used for microarray analysis and the signals are then nonlinearly decomposed to identify the pertinent genetic elements precisely thus measuring clone fitness (Lynch et al., 2007). This method was used to analyze *E. coli* harboring a plasmid-based genomic library stressed with 1-naphthol in batch and in cultures with increasing stressor concentration (Gall et al., 2008). They decomposed cell fitness into two specific traits, namely growth rate and lag time, and were able to map genes to each one in their analysis.

Transgenic libraries can be also constructed where the genome of one organism is utilized in library construction and then screened in a different organism. A library of *Pichia stipitis* DNA was expressed in *S. cerevisiae* and was used to identify genes allowing for growth on xylose (Jin et al., 2005). Upon confirming that *XYL3* allows for increased xylose assimilation, the library was expressed in a *XYL1 XYL2 XYL3* background to identify a strain exhibiting 100% faster growth rate and 70% increase in ethanol production from xylose.

Furthermore, genomic libraries have been used to identify genes or fragments that cause growth inhibition under any defined growth condition (Akada et al., 1997; Boyer et al., 2004; Sopko et al., 2006). Overexpression of most toxic genes resulted in phenotypes different from known deletion mutant phenotypes (Sopko et al., 2006), and was thus concluded that overexpression phenotypes likely reflect regulatory imbalances rather than disruption of protein-complex stoichiometry. The implication is that the two approaches (deletion vs. overexpression libraries) will likely identify different genes for a desirable phenotype. A variation of this approach can partially overcome a key criticism of the library-based methods, namely that they cannot capture interactions among genes in the development of a phenotype. In this method (Kroll et al., 1996), termed SDL for “synthetic dosage lethality”, a cloned “reference” gene (or in its generalized version, a library of genes) is/are inducibly overexpressed in a library of mutant strains carrying potential “target” mutations. The interaction does not need to be lethal, in fact quite the opposite, and thus, this approach can identify a broad spectrum of positive or negative interacting mutations. This approach has been also used in a reverse fashion (Sopko et al., 2006), whereby a genomic library is expressed in a cell mutant for the multifunctional cyclin-dependent kinase (*cdk*) Pho85p, whose full substrate/target repertoire remains unknown. Following selection, the gene set was enriched for Pho85p targets and identified the yeast transcription factor Crz1p as a novel substrate. Thus, this approach can be used to identify genes regulated by specific signaling pathways.

8.4.3. Metagenomic libraries

It has been estimated that there are 10^{30} microbes in the environment (Turnbaugh and Gordon, 2008) and 10^{31} phage particles that can shuttle genomic information between species (Dinsdale et al., 2008). However, the grand majority of bacteria and other microbes cannot be cultured and thus the communities cannot be reproduced in the laboratory (Schmeisser et al., 2007). Metagenomics aims to study the dynamic relationships available in a specific community and determine the interactions and processes that allow for survival in that particular environment. To do this, the entire gene pool available is either sequenced, or cloned into vectors (BACs or cosmids) and screened in suitable hosts for specific traits. Metagenomic libraries can be very useful in creating tolerant phenotypes by identifying novel genes from organisms that thrive in challenging environments or harsh

conditions, such as glyphosate stress (Jin et al., 2007). Glyphosate, a strong herbicide, is an analogue of phosphoenolpyruvate and a competitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). DNA extracted from soil exposed to the herbicide for over 15 years was utilized to construct a metagenomic library which was screened in an *E. coli aroA* knockout (Jin et al., 2007). One novel gene identified in this study exhibited higher resistance to glyphosate up to 150 mM. Similarly, an *E. coli* metagenomic library using DNA isolated from an “industrial effluent treatment plant sewage” was constructed and screened for resistance to arsenate (Chauhan et al., 2009). A novel arsenate tolerance gene that increased wild-type *E. coli* resistance to sodium arsenate six times was discovered in the study. Thus, similar approaches can be used for developing strains for applications in bioremediation.

It is often necessary to enrich for genomes, and techniques such as GC or bromodeoxyuridine enrichment, isotope labeling or enrichment based on a metabolite have been used (Gabor et al., 2007; Schloss and Handelsman, 2003; Schmeisser et al., 2007). For efficient library screening, it is necessary to have the inserts also be expressed in the host. It is also possible to include promoters in the plasmid libraries to ensure transcription and minimize the number of genes which are not getting expressed. *E. coli* is typically used as a host due to the ease of transformation and expression of multiple transgenic genes, but other hosts such as *B. subtilis*, *Streptomyces lividans*, *P. putida*, and *Rhizobium leguminosarium* have been also used (Gabor et al., 2007; Patnaik, 2008).

8.5. Promoter and IR libraries

The pioneering work in developing selectable promoter libraries with synthetic diversity was carried out by Horwitz and Loeb (1986); they developed large promoter-sequence diversity of *E. coli* promoters by replacing 19 base pairs at the –35 promoter region with random DNA sequences thereby generating 3×10^{11} replacement sequences. Screening this large library of promoters, they identified several functional promoter sequences, including two which are stronger than the original promoter. In follow-up work, Horwitz et al. (1989) anticipated the use of such libraries in the development of whole-genome approaches for metabolic and whole-cell engineering. Methods to create sequence diversity have since expanded from random mutagenesis to cassette mutagenesis (Borrego et al., 1995) and later error-prone PCR (Alper et al., 2005). A library of 38 synthetic *L. lactis* promoters was constructed by randomizing the sequences of the separating spacers, while keeping constant the known consensus promoter sequences (Jensen and Hammer, 1998). The screening of these promoters has shown that 400-fold activity changes in small incremental steps can be obtained. Meynial-Salles et al. (2005) developed and screened a larger and more diverse library of chromosomal intergenic regions (IR), which include the promoters upstream of coding genes. This library included diversity not only in promoter sequences, but also diversity in RBS (ribosome binding site) and start codon sequences, thus allowing selection not only for promoter strength, but also for mRNA stability due to altered RBS and start-codon sequences. They demonstrated that this approach can be used to select not only the optimal expression of a gene or genes for growth but also for product formation. This approach was further pursued later by Pflieger et al. (2006). More broadly randomized promoters, by error-prone PCR, were used by Stephanopoulos and coworkers for selecting desirable phenotypic traits in both *E. coli* and *S. cerevisiae* (Alper et al., 2005; Santos and Stephanopoulos, 2008). Although no reports using IR and promoter libraries have been published for generating tolerant phenotypes, the potential

here for such applications is large and remains to be explored. For example, tuning and optimizing the timing and expression level of *groESL* may impart a larger tolerance level to butanol in *C. acetobutylicum* (Tomas et al., 2003) while simultaneously optimizing growth and butanol productivity.

9. Model organisms & their tolerance characteristics

In this section we briefly summarize key characteristics of important industrial organisms related to their stress response and tolerance. While some information that has already been discussed is again briefly presented, the goal here is to present an organism-based discussion of tolerance, aiming to facilitate the comparative analysis of stress response and tolerance in key microorganisms.

9.1. *Pseudomonas putida*, the model organism of enhanced solvent tolerance: a future host for solvent production?

P. putida strains are among the most solvent-tolerant bacteria known, with high tolerance to toluene, butanol, n-decane, n-octane, n-heptane, styrene, p-xylene, diethylphthalate, and several other chemicals (Inoue and Horikoshi, 1989; Ramos et al., 1995; Ruhl et al., 2009; Weber et al., 1993). Many factors contribute to the hardy nature of this organism. Different *P. putida* strains have been reported to grow on toluene, via the benzyl alcohol pathway (for *P. putida* Idaho) or the toluene-dioxygenase pathway (for *P. putida* DOT-T1E) (Cruden et al., 1992; Ramos et al., 2002, 1995). However, it was conclusively demonstrated that knockouts of the relevant genes for toluene metabolism do not diminish tolerance, thus decoupling the effects of metabolism on the tolerance phenotype (Mosqueda et al., 1999). In addition to altering the composition of the cell membrane (Heipieper and de Bont, 1994), *P. putida* also utilizes *cis*-to-*trans* isomerization to increase the ratio of trans-unsaturated fatty acids in the presence of toluene (Weber et al., 1994). To stabilize the membrane under solvent stress, the phospholipid headgroup composition is altered under toluene and ethanol stress, whereby phosphatidylethanolamine (PE) decreases, whereas diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) increase (Weber and de Bont, 1996). Furthermore, *P. putida* exhibits active efflux of toluene in an energy dependent system to discharge toxic solvents from the cell (Isken and de Bont, 1996; Ramos et al., 1998).

9.2. *Escherichia coli*

The relatively high acid tolerance of *E. coli* has been discussed, and efforts have been recently reported to further engineer its tolerance for specific acids (Warnecke et al., 2008). In *E. coli*, the proteins coded by the *acrAB* operon form a multi-drug efflux pump with TolC, which is highly expressed in solvent-tolerant mutants (Aono, 1998). The AcrAB-TolC system actively pumps toxic solvents from the cytoplasm into the extracellular space to increase tolerance. *E. coli* cell membranes undergo stress related changes that differ based on the solvent. An initial response to solvent stress is the *cis*-to-*trans* isomerization of phospholipids (Ramos et al., 2002), which allows for denser packing of the phospholipids to minimize the fluidizing effects of solvents. To further counteract the effects of ethanol on the cell membrane in the long term, the ratio of unsaturated to saturated fatty acids increases, and so does the length of phospholipid chains (Buttke and Ingram, 1980; Ingram, 1977; Weber and de Bont, 1996). Higher alcohols (C6–C10) and phenol induce an opposite effect,

namely a decrease in the ratio of unsaturated to saturated fatty acids (Weber and de Bont, 1996). Furthermore, multiple chaperones such as DnaJ–DnaK and GroES–GroEL, as well as heat-shock proteins like Hsp33 are involved in stress tolerance, and may have an active role in increasing solvent tolerance, as well (Lund, 2009; Winter et al., 2005). *PspA* is also expressed in *E. coli* and is involved in solvent stress response (Kobayashi et al., 1998). As discussed, this Psp member minimizes proton leakage under stress to help restore cellular functions (Kobayashi et al., 2007), and helps maintain an active membrane potential.

9.3. *Saccharomyces cerevisiae*

Ethanol causes an increase in the membrane fluidity of *S. cerevisiae* and destroys functional membrane structures. Yeast cells have developed response mechanisms to tolerate such alcohol stresses. It is well established that the levels of unsaturated fatty acids and ergosterol increase during ethanol stress to counteract the effects of ethanol (Swan and Watson, 1998; You et al., 2003). Ergosterol is the most abundant sterol in *S. cerevisiae*: it facilitates higher ethanol tolerance by increasing membrane rigidity (Ding et al., 2009). Unsaturated fatty acids, such as oleic acid, antagonize the effects of ethanol on membranes (Ding et al., 2009). A fourfold increase in the ratio of oleic acid to palmitoleic acid in the presence of ethanol in an ethanol-tolerant strain was reported (You et al., 2003). The incorporation of oleic acid into cell membranes decreases fluidity, thus compensating for the fluidization elicited by ethanol. Furthermore, trehalose accumulates in yeast cells under stress (Attfield, 1987; Vianna et al., 2008) and increases tolerance by stabilizing the cell membrane and preventing the accumulation of misfolded membrane proteins (Ding et al., 2009). Other HSPs and chaperones are also involved in tolerance (Galeote et al., 2007; Piper, 1995), but the particular role of chaperones to ethanol tolerance is not yet well understood (Ding et al., 2009). Transcriptional regulators, including global transcription factors (Alper et al., 2006) and proteins with zinc-binding motifs (MacPherson et al., 2006), also alter the cellular response to tolerate ethanol stress. Shi et al. successfully utilized WGS in *S. cerevisiae* strain SM-3 to enhance thermotolerance, ethanol productivity and ethanol tolerance (Shi et al., 2009). They reported strains that exhibit tolerance to 25% v/v ethanol with viabilities up to 60% compared to the parent strain. As discussed, WGS was also used to generate strains resistant to biomass hydrolysates. Regarding acid tolerance, examples have already been discussed, such as, e.g., the case where lactate tolerance was improved in an engineered strain to produce ascorbic acid, which is a well-known ROS scavenger (Abbott et al., 2009).

9.4. Solventogenic *Clostridia* and butanol

Butanol is desirable as either a commodity chemical, biofuel or a gasoline extender. The traditional industrial process was a batch fermentation of solventogenic *Clostridia* such as *C. acetobutylicum*. Among the key factors responsible for the poor process economics are the low butanol titers in the product stream (Dadgar and Foutch, 1988; Lenz and Moreira, 1980; Linden et al., 1985; Marlatt and Datta, 1986). Low butanol titers are partially due to the low tolerance of these organisms to butanol: final butanol concentrations rarely exceeded 12–13 g/l (Linden et al., 1985; Marlatt and Datta, 1986). The log *P* of n-butanol is 0.8, making it one of the most toxic solvents (Bruce and Daugulis, 1991). Relatively few butanol-tolerance studies have been reported. A *Bacillus* strain isolated from mangrove sediment was reported to grow in 2% (v/v) butanol with a 72% reduction in growth rate (Sardesai and

Bhosle, 2002). *L. brevis* was reported to grow in the presence of 2% (v/v) butanol, exhibiting limited growth in 3% (v/v) butanol (Knoshaug and Zhang, 2009). It was also reported that *E. coli* and *P. putida* can survive 1% butanol (Nielsen et al., 2009), but a separate report shows data whereby adapted *P. putida* grows in the presence of up to 6% (v/v) butanol (Ruhl et al., 2009). As already discussed, butanol toxicity in solventogenic *Clostridia* is attributed to its chaotropic effect on the cell membrane (Bowles and Ellefson, 1985; Vollherbst et al., 1984). At high concentrations, butanol inhibits active nutrient transport, membrane bound ATPases, and glucose uptake; it partially or completely abolishes the membrane ΔpH and $\Delta\psi$, and lowers the intracellular pH and ATP concentration. Lipids from exponential-phase cultures contain approximately 58% saturated acyl-chains while stationary phase lipids contain approximately 77% saturated chains. This alteration occurs in order to counteract the effect of solvents on the membrane fluidity and functionality. Exponential growth cultures grown in 0.5% and 1.0% (v/v) butanol have saturated chain contents of 65% and 73%, respectively, but cultures with 1.5% butanol (ca. 14 g/l) did not grow at all (Ounine et al., 1985; Vollherbst et al., 1984). In contrast, acetone and ethanol are not inhibitory to growth in concentrations up to 20 g/l (Ounine et al., 1985).

Solvent-tolerant strains of *C. acetobutylicum* have been generated through serial enrichment of liquid cultures with butanol. Strains SA-1 (Lin and Blaschek, 1983) and G1 (Soucaille et al., 1987) were developed in this manner and were tolerant (no more than 50% reduction in specific growth rate) in 15 and 18 g/l butanol. The increased tolerance did not result in a proportional increase in butanol production; butanol production in SA-1 improved from 12.6 to 13.9 g/l and G1 from 10.5 to 13.0 g/l. A *C. beijerinckii* strain BA101 (Annous and Blaschek, 1991) produced by mutagenesis produces 19 g/l butanol (Formanek et al., 1997). In addition to these methods, metabolic engineering approaches have been also reported to generate strains with increased butanol tolerance. Our laboratory has generated a strain (by inactivating the so-called *solR* gene; CAP0162), which produces 250 mM (18.5 g/l) butanol, 160 mM (9.3 g/l) acetone and 47 mM (2.2 g/l) ethanol (Harris et al., 2001). Another strain generated by the *buk* inactivation and *aad* (CAP0162) overexpression produces 230 mM (17 g/l) butanol (Harris et al., 2000). A third strain, generated by overexpressing *groESL*, as already discussed, produces butanol up to 231 mM (17 g/l) (Tomas et al., 2004, 2003).

9.5. *Lactobacilli* the alcoholotolerant: hosts for solvent production?

Lactobacilli include some of the most ethanol, butanol and generally alcohol-tolerant organisms known (Couto et al., 1997; G-Alegria et al., 2004; Gold et al., 1992; Knoshaug and Zhang, 2009). G-Alegria et al. (2004) demonstrated that *L. plantarum* grows at pH 3.2 with up to 13% ethanol, but did not investigate the genes imparting this superior tolerance. It was reported that *Lactobacilli* are also tolerant to biomass hydrolysates (Knoshaug and Zhang, 2009). WGS was also used to improve acid tolerance in *Lactobacilli* (Patnaik et al., 2002; Zhang et al., 2002). Several *Lactobacillus* genomes have been sequenced, and several more are currently sequenced. Although not all alcohol-tolerant strains (e.g., *L. hilgardii*) have been sequenced and annotated, the genome for *L. plantarum* has (Kleerebezem et al., 2003).

9.6. *Zymomonas mobilis*, an important ethanol-tolerant ethanologen

Z. mobilis, a Gram[−] ethanologenic bacterium, can ferment sucrose, glucose and fructose to ethanol via the Entner–Doudoroff

(ED) pathway with high specificity with minimal glucose (~2%) being incorporated into cell biomass (Hermann et al., 2006; Olsson and Hahn-Hagerdal, 1996; Swings and De Ley, 1977). It has a superior ethanol to glucose yield, whereby 95% of glucose is converted to ethanol and CO₂, and this, combined with excellent cell productivities, has made *Z. mobilis* an important organism for ethanol production. It can grow in media with high glucose concentrations and has a high tolerance (for a prokaryote) to ethanol (10% v/v) (Swings and De Ley, 1977). *Z. mobilis* membranes contain over 75% vaccenic acid (18:1) in polar lipids, and membrane composition does not change under ethanol stress, thus demonstrating that the microbe evolved for high tolerance (Carey and Ingram, 1983). The cytoplasmic membranes in *Z. mobilis* contain hopanoids (such as diploptene, diptoptol, and bacteriohopanetetrol ether) that stabilize the phospholipid bilayer to resist ethanol fluidization; inhibition hopanoid synthesis decreases ethanol tolerance (Hermann et al., 2006). In addition, overexpression of HSPs, including DnaK, GroEL and GroES has been linked to ethanol tolerance (Barbosa et al., 1994; Michel, 1993). Its genome has been recently sequenced (Kouvelis et al., 2009), thus raising the expectation that a molecular understanding of the mechanisms leading to high ethanol tolerance can be better understood and possibly applied to other organisms. A *Z. mobilis* strain was engineered for acetic-acid tolerance by overexpressing an *E. coli* gene (*cbpA*) coding a 24 amino acid peptide which has proton-buffering capacity (Baumler et al., 2006); the mechanism of this tolerance is not known.

9.7. *Deinococcus radiodurans*: tolerance to radioactive chemicals and solvents

D. radiodurans exhibits remarkable resistance to ionizing radiation (Cox and Battista, 2005) and can repair extensive DNA damage, including the complete reassembly of its genome from fragments as small as 20–30 kb (Slade et al., 2009). Thus, *D. radiodurans* is recognized as an important organism for bioremediation applications in radioactive sites. Lange et al. (1998) cloned the *tod* genes under a constitutive promoter to functionally express toluene dioxygenase (TOD) in *D. radiodurans* and oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene, and indole in highly radioactive environments. *D. radiodurans* strains are also naturally resistant to solvents, notably toluene and trichloroethylene (Lange et al., 1998). Recently, it was shown that another *Deinococcus* strain, *D. geothermalis* T27, exhibits high tolerance to numerous solvents spanning a wide range of log P values including butyl acetate (1.8), ethyl acetate (0.7), toluene (2.5), and benzene (2.0) (Kongpol et al., 2008). The mechanisms involved in this solvent tolerance are not known, but the addition of glucose or fructose increased its solvent tolerance (Kongpol et al., 2008).

10. Closing remarks

We have reviewed a large body of literature on how cells respond to solvent, carboxylic-acid and related chemical stress, and on efforts to adapt or engineer tolerant strains to these chemicals. These classes of chemicals cover a very large fraction of applications encountered in bioprocessing whether for biofuel and chemicals production, biocatalysis or bioremediation, and include the most significant inhibitory chemicals found in biomass hydrolysates. We have shown that there are some general classes of genes or programs that can be engaged and engineered to impart chemical tolerance such as HSP proteins and specialized stress sigma factors and their regulons (Box 2). With recent advances in genomics and systems biology, it is likely that

an evolved or *ab initio* designed synthetic or semi-synthetic HSP system or stress regulon to deal with pan-chemical stress and tolerance is within reach in the next few years. To enable this, one could envision semi-synthetic, “designer” organisms generated by systematic genome enlargements and re-arrangements based on a platform organism such as *E. coli*, a *Lactobacillus*, a *Pseudomonas*, or a yeast species. While non-trivial, this is in principle possible, and may be the basis for generating eventually totally synthetic organisms suitable for specialized bioprocessing applications.

For solvents, it is now clear that engineering the composition of the cell membranes and wall to better withstand the chaotropic effects of solvents is an excellent target for advanced metabolic engineering. One could in fact argue for studies to *ab initio* design cell membranes that would tolerate high levels of solvents similar or even higher than those tolerated by species of *Pseudomonas*, *Lactobacillus*, *Rhodococcus*, *Deinococcus*, and *Nocardioideis*, or of yeast genera like *Kluyveromyces* or *Pichia*. Similarly, one could envision a “designer” system of efflux pumps to impart specialized solvent tolerance. Every program that has been reviewed as possibly imparting tolerance to solvents or acids or any other chemical could be interrogated with modern genomic and systems biology tools (see, e.g., Fig. 2), analyzed and re-designed for better performance, and then engineered into organisms in the context of a synthetic or semi-synthetic “designer” strain.

It is also desirable to improve current genomic tools and develop new ones towards the goal of generating synthetic or semi-synthetic programs and organisms. For example, genomic and metagenomic libraries have some limitations that need to be addressed. Plasmid-based libraries are limited by the insert size they can harbor and therefore cannot readily capture interactions among multiple genes of a program distantly located on a chromosome. Furthermore, for metagenomic or heterologous libraries, the hosts selected for library expression will influence the screening outcome. This is because heterologous genes are not likely to be recognized by the transcription machinery of the host, and genes that will not get expressed during the screening, will not be enriched. One could envision designing a platform organism with multiple but compatible transcription machineries to deal with this issue.

It is clear that much progress has been made over the last 40 years in understanding and ameliorating chemical stress, and that recent genomic and systems biology efforts have accelerated this understanding. Given this large body of knowledge and the renewed interest in the problem of chemical tolerance for bioprocessing applications, future developments should anticipate dramatic strain improvements. Five to ten years from now, bioprocessing strains are likely to look very different from their natural predecessors, if not totally synthetic.

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