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Engineering inhibitor tolerance for the production of biorenewable fuels and chemicals

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Abstract

Metabolic Engineering has enabled the production of biorenewable fuels and chemicals from biomass using recombinant bacteria. The economic viability of these processes is often limited by inhibition of the biocatalyst by the metabolic product, such as a carboxylic acid or alcohol, or by contaminant compounds in the biomass-derived sugars, such as acetic acid or furans. Historically, selection-based methods have been used to improve biocatalyst tolerance to these inhibitors. But recently, genome-wide analysis has been used to both identify the mechanism of inhibition and reverse engineer inhibitor-tolerant strains, enabling the rational, predictive manipulation of bacteria in order to increase inhibitor tolerance. Here we review recent work in this area, particularly in relation to carboxylic acids, furfural and butanol.
Highlights

- Inhibition of bacterial metabolism hinders production of biorenewable compounds
- Transcriptome analysis can be used to identify the mechanism of bacterial inhibition
- Randomly-selected tolerant strains can be reverse engineered to find key mutations
- When the mechanism of inhibition is known it can be rationally alleviated
- Efflux pumps and alteration of the cell membrane are increasingly important

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Introduction

Metabolic Engineering has enabled the predictive, rational redesign of bacterial metabolism for a variety of desired properties, such as increased substrate range [1], altered product profile [2], fine-tuned enzyme function [3] and de novo pathway design [4]. However, as we strive to produce fuels and chemicals at high yields and titers, biocatalyst inhibition by the product becomes an increasingly vexing problem. For example, next-generation biofuels, such as butanol, and drop-in replacements for petroleum-derived bulk chemicals, such as styrene, are toxic to the biocatalyst [5-7]. Hemicellulose hydrolysate and pyrolysis-derived bio-oil are attractive forms of biomass-derived sugars, but their utilization as fermentation feedstocks is hindered by the presence of compounds that are inhibitory to the biocatalyst [8-11]. This toxicity means that the biocatalyst growth and production of the desired metabolic product are limited. Thus, both ends of biocatalyst metabolism are impacted by inhibition: the utilization of cheap, biomass-derived sugars is limited by inhibitory compounds in the feedstock, while commercially viable production of biorenewable fuels and chemicals is limited by product toxicity (Figure 1).

Sometimes this toxicity can be mitigated by removal of the inhibitory compound by pre-treatment of the feedstock [12], continual removal of the inhibitory product [13,14] or production of a chemical precursor of the desired product that is easier to remove or is less inhibitory [15]. However, this approach is not feasible with all compounds and may result in increased process cost. This review focuses on the complementary approach of increasing the tolerance of the biocatalyst of these inhibitory compounds.

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The most successful strategies for improving biocatalyst tolerance utilize a selection-based approach, such as metabolic evolution [16,17], enrichment of expression libraries [18,19], mutation of global transcription machinery [20], hybridization of the biocatalyst with another biocatalyst that has tolerance of the target compound [21,22], and enrichment of metagenomic libraries [23]. In these cases, bacteria that acquire a mutation or gene that confers tolerance grow faster in the presence of the inhibitor and can be selected by growth-based assays. However, our current wealth of knowledge regarding the standard biocatalysts, such as \textit{Escherichia coli} and \textit{Saccharomyces cerevisiae}, and advanced DNA manipulation tools means that we are now beginning to have the ability to engineer tolerance with the same rational, predictive approach that we use for engineering metabolism.

Here we describe recent advances in rational engineering for inhibitor tolerance. Rational engineering of tolerance generally requires a known mechanism of inhibition. Genome-wide analysis during inhibitor challenge and reverse engineering of inhibitor-tolerant strains are both effective methods for identifying these inhibition mechanisms (Figure 2). We describe three model inhibitor types: furans, carboxylic acids and alcohols. Furans, such as furfural, are contaminants in sugars derived from lignocellulosic biomass. Short-chain carboxylic acids, such as acetic acid, are feedstock contaminants, while longer-chain acids, such as lauric acid, are biorenewable products. Alcohols, such as butanol, are next-generation biofuels.

**Identification of the mechanism of inhibition by omics analysis**

While the scientific literature is often rich in information regarding the response of specific enzymes to inhibitors, a genome-wide view is invaluable for determining the critical effect responsible

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for growth inhibition. Transcriptome and proteome studies provide this view by simultaneously measuring all expressed transcripts and proteins, respectively.

Transcriptome studies of *E. coli* showed that furfural’s toxicity can be at least partially attributed to limitation of hydrogen sulfide production, due to depletion of NADPH by furfural reduction [24]. Hydrogen sulfide is a required for biosynthesis of cysteine, which is in turn needed for methionine biosynthesis. Studies in yeast have also shown a critical role of NADPH in furfural tolerance [25]. There are certainly additional mechanisms of furan toxicity beyond NADPH depletion, as evidenced by the fact that supplementing with cysteine is only helpful at low furfural concentrations [24]. However, identification of these additional toxicity mechanisms is confounded by the masking effect of low-level toxicity mechanisms. Additional analysis would be useful in identifying the higher-level toxicity mechanisms.

Transcriptome analysis of octanoic and decanoic acid challenge in *S. cerevisiae* revealed that these two carboxylic acids both activate oxidative stress genes [26]. This analysis also showed that a critical component of the detoxification response is expression of transporters than can aid in acid expulsion, such as Pdr12p and Tpo1p. Abbott *et al* also used transcriptome analysis to investigate carboxylic acid toxicity in *S. cerevisiae* using benzoate, sorbate, acetate and propionate as model compounds [27]. While a core set of 14 genes were upregulated during exposure to all acids, the link between these genes and the mechanism of inhibition is not yet clear. However, it was clear that genes associated with the cell wall were overrepresented in the set of perturbed genes, indicating an interaction between carboxylic acids and this critical cell structure. Finally, a transcriptome analysis of the acetic acid response in *S. cerevisiae* identified the HRK1 protein kinase as a critical regulator of

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transporter activity; in its absence, intracellular acetate accumulation was increased [28]. These results highlight a common theme in inhibitor tolerance: altered expression of transporters can play a major role in inhibitor tolerance, presumably by relocating the toxic compound from inside the cell to the external environment.

Recently, metabolic flux analysis has been used to reveal perturbations in *E. coli*’s central metabolism during challenge with octanoic acid (*Fu, Yoon and Shanks, manuscript in preparation*). This analysis suggested that the flux through pyruvate dehydrogenase and the TCA cycle are decreased during inhibition by octanoic acid, possibly due to an imbalance in the redox ratio due to membrane damage.

While ethanol toxicity has been extensively studied, butanol toxicity is a relatively new issue. Comparative studies have shown that the mechanism of butanol toxicity is unique compared to other molecules such as ethanol [29]. Brynildsen *et al* used transcriptome analysis to probe the mechanism of isobutanol toxicity and concluded that membrane damage is a key component of isobutanol toxicity, due at least in part to disruption of the electron transport chain [7]. This study also showed that the n-butanol and isobutanol transcriptional responses were qualitatively similar, while confirming that the ethanol response was quite different. Screening of an expression library identified several membrane lipoproteins as important for butanol tolerance, further emphasizing the importance of the cell membrane to butanol tolerance [19]. Additional mechanisms of butanol toxicity are nicely summarized in a recent review by Ezeji *et al* [6].

Reverse Engineering Tolerant Strains

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While metabolic evolution has long been a key technique for strain improvement, it is only recently that DNA sequencing has become affordable enough to enable whole-genome sequencing and subsequent identification of the key mutations enabling the tolerance phenotype in evolved strains. Transcriptome analysis and metabolic flux analysis have also proven to be effective in enabling reverse engineering.

A transcriptome-based approach was used to reverse engineer furfural-tolerant *E. coli*, leading to identification of YqhD as the major NADPH-dependent furfural reductase in *E. coli* [17]. During furfural challenge, this enzyme drains valuable NADPH away from cysteine biosynthesis, limiting biocatalyst growth [17,24]. Sequence analysis revealed that an IS10 insertion within the *yghC* regulator silenced *yqhD*, where this silencing ultimately spares NAPDH for biosynthesis [30]. Thus, this reverse engineering study revealed not only that YqhD is the major *E. coli* furfural reductase but also that YqhC regulates *yqhD*. This highlights the contribution that reverse engineering can make to our understanding of even well-characterized biocatalysts like *E. coli*.

As opposed to the analysis of inhibitor-tolerant strains, the reverse engineering of strains with increased inhibitor sensitivity can also be informative. Such was the case for a gene disruption library of *S. cerevisiae* with increased furfural sensitivity [31]. Mutations of genes within the pentose phosphate pathway were shown to increase sensitivity to furfural, and the subsequent increase in tolerance conferred by overexpression of *zwf1* supported this result [31]. These results are surprisingly consistent with the *E. coli* results, as the pentose phosphate pathway is the major source of NADPH.

An evolved acetate-tolerant strain of *Zymomonas mobilis* was reverse engineered by both genome and transcriptome analysis [32]. It was shown that increased expression of sodium-proton

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antiporter nhaA, due to deletion of an unspecified regulator, confers the evolved phenotype. This again shows that altered expression of a transporter can impact inhibitor tolerance.

Whole-genome sequencing was used to identify the nature of and then reconstruct isobutanol tolerance in isobutanol-producing E. coli [33]. Each of the 27 mutations detected in the evolved isobutanol-tolerant strain was reconstructed in the parent strain in order to test its contribution to the tolerance phenotype, with the finding that 5 key mutations are responsible for tolerance. The fact that one of the key mutated genes, yhbJ, is involved in regulation of synthesis of a cell membrane component, again highlights the relationship between isobutanol toxicity and the cell membrane.

Metabolic flux analysis of n-butanol tolerant strains of Pseudomonas putida revealed that tolerant strains had decreased maintenance cost [34]. This observed decrease in TCA cycle use and energy production was interpreted as an adaptation of the cell membrane to butanol toxicity, again emphasizing the importance of this complex yet fragile structure in alcohol tolerance.

**Rational Engineering for Tolerance**

When a mechanism of inhibition is known, it can be addressed by rational engineering efforts, similar to the way that biocatalyst metabolism can be rationally and predictably engineered.

As described above, depletion of NADPH pools by the NADPH-dependent furfural reductase YqhD is at least partially responsible for furfural-mediated growth inhibition [17]. One rational approach for mitigating this effect is to spare NADPH by silencing yqhD [17]. Another approach is to convert abundant NADH to NADPH via the transhydrogenase enzyme [24]. Yet another rational approach is to reduce furfural to the less-toxic furfuryl alcohol while sparing NADPH by overexpressing an NADH-
dependent furfural reductase. This approach was implemented by Wang et al: overexpression of the NADH-dependent FucO in the absence of yqhD increased furfural tolerance by 50% (Table 1) [35].

As described above, many studies have identified the relationship between butanol toxicity and the bacterial cell membrane. This lipid bilayer serves as an important barrier that separates fragile bacterial components from harsh environmental conditions as well as the scaffold for the electron transport chain. Therefore, membrane fluidity and integrity are critical for effective cell function, as reviewed in [36]. The functionality of the plasma membrane is largely determined by its composition, where composition includes such variables as saturated vs unsaturated fatty acids, straight-chain vs cyclopropanated fatty acids, and cis vs trans unsaturated fatty acids.

Given this importance of the cell membrane and evidence that this membrane is damaged by alcohols, Luo et al improved E. coli’s tolerance of ethanol by overexpressing the fabA dehydrase [37]. This increased both the saturated fatty acid content and ethanol tolerance (Table 1). This rational engineering of the membrane composition raises some intriguing possibilities. Some organisms produce very long or odd-numbered fatty acids chains, with some chains being up to 35 carbons in length [38]. Other organisms produce fatty acid with multiple cyclopropyl groups or use highly-strained “ladderane” fatty acids to form intracellular compartments [39]. As more evidence accumulates that desirable biorenewable fuels and chemicals damage the bacterial membrane, the motivation for engineering membrane resilience and integrity will increase and we may start to see utilization of these unique fatty acids.

As mentioned above, another common theme in inhibitor tolerance is the use of transporters and to remove the toxic compound from inside the cell. With this fact in mind, a competition-based
strategy was used to screen 43 distinct efflux pumps for their ability to confer tolerance to biofuel-type compounds in *E. coli*. Except for n-butanol and isopentanol, this library of efflux pumps was effective for improving the tolerance to all other studied biofuels candidates. Additionally, certain pumps that improved the tolerance to the biofuel also increased production of that biofuel; specifically, use of a limonene efflux pump significantly increased limonene production (Table 1) [40].

**Conclusion**

Here we have described some recent advances in both the identification of the mechanism of bacterial inhibition by toxic compounds as well as the rational engineering of tolerance itself. Given the desire to biocatalytically produce next-generation biofuels and drop-in replacements of bulk chemicals, this is an increasingly important problem. It is also apparent that strategies need to be developed for increasing the resilience of the bacterial cell membrane for these toxic compounds.

However, it is not yet clear if increasing tolerance will always result in an increase in biocatalyst performance. While identification of a limonene efflux pump resulted in an increase in limonene production [40], reverse engineering and reconstruction of isobutanol tolerance unfortunately did not result in an increase of isobutanol production [33]. This is a clear demonstration of the difficulty of designing the appropriate selection strategy for selection-based strain improvement. When the biocatalyst produces the inhibitory compound during stationary phase, selection for inhibitor tolerance during log phase may not have the desired positive impact on productivity. Thus, the development of additional selection techniques, in addition to the rational engineering of tolerance, would be extremely useful in this area.

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As our ability to analyze the deluge of data acquired by next-generation DNA sequencing improves, it is expected that the number of successful reverse engineering projects will increase. The knowledge acquired from these projects will enable additional rational engineering efforts and hopefully, an ultimate gain in our ability to produce biorenewable fuels and chemicals from biomass in place of petroleum.

Acknowledgements

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5. McKenna R, Nielsen D: **Styrene biosynthesis from glucose by engineered *E. coli.*** *Metabolic Engineering* 2011, in press.


A comprehensive review of the recent data acquired regarding isobutanol toxicity in a variety of biocatalyst and strategies for mitigating that toxicity. Both strain improvement and selective product removal are discussed.


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Transcriptome analysis was used to investigate isobutanol toxicity in *E. coli*. It was concluded that disruption of the electron transport chain, possibly due to membrane damage, is a key stressor to the bacteria.


*10. Mills TY, Sandoval NR, Gill RT: **Cellulosic hydrolysate toxicity and tolerance mechanisms in Escherichia coli.** Biotechnology for Biofuels 2009, 2. A timely review of the inhibitors associated with biomass hydrolysate and mechanisms for overcoming that resistance when *E. coli* is used as the biocatalyst.


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Both genome and transcriptome studies are used to reverse engineer acetate-tolerant derivatives of *Zymomonas mobilis*, ultimately identifying an acetate transporter as a key component of the tolerance phenotype.


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The authors performed a rigorous sequenced-based analysis of an isobutanol-tolerant derivative of isobutanol-producing *E. coli* and ultimately rationally reconstructed the tolerance phenotype. Unfortunately, the acquisition of isobutanol tolerance did not increase isobutanol tolerance.


Based on the known mechanism of furfural's toxicity, the authors devised and implemented a rational method for both increasing furfural tolerance and ethanol production in the engineered strain.


The authors use recombinant expression of two fatty-acid modifying enzymes to rationally alter the composition of *E. coli*’s cell membrane and therefore tolerance to ethanol.


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The authors screen many bacterial efflux pumps in *E. coli* for the ability to increase tolerance to a variety of biofuel-type compounds. Not only were they successful in identifying many exciting efflux pumps, but they clearly demonstrated that enabling increased efflux also enables increased tolerance.
Figure 1: Biocatalyst inhibition by contaminant compounds in biomass-derived sugars and biorenewable fuels and chemicals limits the attainment of economically viable yields and titers.
Figure 2: Rational engineering for inhibitor tolerance requires a known mechanism of inhibition. Methods for identifying these mechanisms include omics analysis and reverse engineering of evolved strains.
Table 1: Rational engineering of *E. coli* for inhibitor tolerance

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<tr>
<th>Inhibitor</th>
<th>Improvement method</th>
<th>Note</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>Expression of NADH-dependent furfural reductase FucO</td>
<td>Minimum inhibitory concentration increased from 10 to 15 mM</td>
<td>[35]</td>
</tr>
<tr>
<td>Furfural</td>
<td>Expression of membrane-bound transhydrogenase <em>pntAB</em></td>
<td>Increased tolerance to 1.0 g/L</td>
<td>[24]</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Increased saturated fatty acid content in the membrane via expression <em>fabA</em></td>
<td>Increased tolerance to 3%</td>
<td>[37]</td>
</tr>
<tr>
<td>Limonene</td>
<td>Expression of <em>A. borkumensis</em> efflux pump YP_692684</td>
<td>Increased tolerance to 0.03% v/v; increased production from 35 to 55 mg/L</td>
<td>[40]</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>Rational introduction of key mutations acquired during metabolic evolution: ΔtnaA, ΔgatY, ΔacrA, ΔmarCRAB, ΔyhbJ</td>
<td>Increased tolerance to 8 g/L; no increase in productivity</td>
<td>[33]</td>
</tr>
</tbody>
</table>