Engineering membrane and cell-wall programs for tolerance to toxic chemicals: beyond solo genes

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Abstract

Metabolite toxicity in microbes, particularly at the membrane, remains a bottleneck in the production of fuels and chemicals. Under chemical stress, native adaptation mechanisms combat hyper-fluidization by modifying the phospholipids in the membrane. Recent work in fluxomics reveals the mechanism of how membrane damage negatively affects energy metabolism while lipidomic and transcriptomic analyses show that strains evolved to be tolerant maintain membrane fluidity under stress through a variety of mechanisms such as incorporation of cyclopropanated fatty acids, *trans*-unsaturated fatty acids, and upregulation of cell wall biosynthesis genes. Engineered strains with modifications made in the biosynthesis of fatty acids, peptidoglycan, and lipopolysaccharide have shown increased tolerance to exogenous stress as well as increased production of desired metabolites of industrial importance. We review recent advances in elucidation of mechanisms of toxicity and tolerance as well as efforts to engineer the bacterial membrane and cell wall.

Introduction – Microbial metabolite toxicity remains a limiting bottleneck in the production of fuels and chemicals

The repertoire of metabolites that can be produced as commodity or specialty chemicals and biofuels or biofuel precursors through microbial processes is ever expanding, and includes organic acids, alkanes, long-chain alcohols and aldehydes, and free fatty acids [1]. Most industrially attractive chemicals are toxic to microbes, an issue that limits the development of economically feasible bioprocesses [1,2]. Engineering or adapting organisms to tolerate high titers of toxic chemicals under realistic metabolite-production conditions aim to make them capable of producing toxic metabolites faster and at higher titers. Thus, understanding and developing microbial tolerance to a broad spectrum of chemicals is a problem of major industrial significance [1]. It is necessary that tolerant strains are capable of producing the desirable toxic metabolites at higher rates, and titers, and not merely tolerate their toxicity [3,4].

The focus of this review: bacterial membranes and cell wall as they relate to metabolite toxicity

The last 10 years have witnessed a large activity in tolerance engineering, including the use of genetic and genomic screens and methods, and synthetic approaches, including focus on the role and impact of membrane transporters[2,4-11]. With few exceptions, the emphasis has been on deleting or overexpressing one gene/protein at a time, where the improvement in tolerance has been small to modest. Little effort has been placed on designing new or modified cellular programs or systems as a means to combat toxicity. Thus, this review focuses on precisely this possibility with emphasis on the understanding and engineering the bacterial membrane and cell wall at the systems level.

Insights and inspiration for engineering tolerance can come from a cell's natural response to toxicity, the differences between a tolerant mutant and the wild type, and even comparing the response mechanisms between strains and species which have varying degrees of naturally occurring tolerance. Beyond comparisons, use of heterologous sources of genetic material, from pure culture or metagenomic sources, can confer tolerance to a toxicity that the host strain may not be able to attain by manipulating only the endogenous genetic material. These are the guiding principles as we navigate through the literature to understand and engineer the tolerance phenotype.

Native membrane-adaptation mechanisms aim at constant membrane fluidity to combat the impact of toxic environments

Microbial membranes are permeable to small neutral molecules needed for growth (e.g. water, oxygen) as well as waste products for passive expulsion (e.g. carbon dioxide, hydrogen), but create a large free energy barrier to diffusion of large and highly polar/charged molecules (Box 1). In addition to these 'gatekeeper' functions, the ability of the membrane to maintain a chemical potential is crucial for energy generation and other cellular functions. Damage to the cell membrane has long been implicated in the toxicity of various chemicals, with the degree of toxicity correlating to the hydrophobicity[12,13]. Short alcohols and hydrophobic compounds, such as ethanol or butanol, can partition into lipid bilayer membranes[14,15]. These compounds displace water at the membrane/water interface which reduces surface tension. As concentrations and the alcohol carbon chain length rise, the membrane becomes overly fluid, which compromises its integrity[14]. Unsaturated fatty acids allow short alcohols to partition the membrane more than saturated fatty acids, which affects membrane phase behavior[14,16]. Exogenous free fatty acids, common in biodiesel-derived crude glycerol feedstocks, can partition into membranes and have a detrimental effect on growth and fermentation[16-18].

Under changing environmental conditions or stress, such as chemical stress from solvents, acids, and temperature changes, microbes engage several mechanisms for maintaining membrane integrity and fluidity; this is largely done by modifying the fatty acid profile of the phospholipid bilayer, termed the homeoviscous response. Modulating the fatty acid profile is not only important in maintaining membrane stability and integrity, but also for proper function of proteins and cofactors associated with the membrane. Generally, saturated fatty acids increase membrane stability and provide resistance to solvents and high temperatures by packing tightly together due to increasing van der Waals interactions between the acyl chains[19]. In contrast, by breaking up this tightly packed configuration, *cis*-unsaturated acyl chains increase membrane fluidity [19]. Regarding chain length, longer acyl chain lengths lead to an increase in the stability and rigidity of the membrane while shorter chains increase fluidity.

While the specific responses of microbes to exogenous stress vary, strains naturally tolerant or evolved to be so generally maintain constant membrane fluidity. Exogenous challenge with high levels of hydrophobic chemicals generally leads to an increase in acyl chain tail length and lipid packing density in bacteria, archaea, and yeast[20-24]. Low levels of less hydrophobic compounds (e.g. <10 g/L ethanol) lead to no change or an increase in unsaturated fatty acid content and an increased tail length[20,24]. Of those reported in the literature, *C. beijerinckii* is

unique in that it completely abolishes production of unsaturated fatty acids under alcohol stress and greatly increases membrane rigidity to enable it to grow in relatively high levels of alcohol[20].

Besides saturated and unsaturated fatty acids, other types of modification can be also engaged for maintaining membrane fluidity. Cyclopropanated and *trans*-unsaturated fatty acids are discussed below. Additionally, some bacteria do not produce unsaturated fatty acids, but rather produce saturated dimethoxy alkanes (e.g. a strain related to *Eubacterium cylindroides*) or branched chains (a diverse variety), which have a similar density-reducing effect and decrease in abundance when subjected to solvent stress [23,25]. Strictly anaerobic bacteria (e.g. *Clostridium* genus) produce plasmalogens, lipids that have a vinyl ether linking group at the *sn*-1 position of the glycerol as opposed to the ester linking group of a common phospholipid, which are more prevalent during solvent stress[22,25,26]. Very long chain α,ω -dicarboxylic acids, found in certain extremophiles (e.g. *Thermoanaerobacter pseudoethanolicus*), span the bilayer and are found in greater prevalence during alcohol stress and increase membrane stability[20]. These mechanisms to alter membrane fluidity may prove to be a fruitful target for solvent tolerance engineering.

Adapted and evolved strains confer tolerance by changing lipid profile

Adaptation and directed evolution are established methods for enabling increased growth in the presence of toxic chemicals or other unfavorable culture conditions. Table 1 lists select literature studies demonstrating changes in lipid composition in evolved strains compared to the parent, which explain in part or in whole the growth-rate increase. Increases in growth rate are generally associated with strains better maintaining optimal membrane fluidity under stress. There is evidence that there are multiple paths to reach a tolerant strain outcome. For example, under octanoic acid stress, short term adaptation of *E. coli* led to an increased unsaturated to saturated lipid ratio (U/S) and an increased acyl chain length as well as a decrease in the cell surface hydrophobicity, while unadapted cells under octanoic acid stress displayed an increase in membrane fluidity and reduced tolerance[27]. An *E. coli* strain evolved on octanoic acid showed increased unsaturated fatty acid content and produced 5-fold higher titers of octanoic acid compared to the parent strain[28]. Likewise, an *E. coli* mutant, EcNR1 G3.2, more tolerant to isobutanol, showed increased U/S during growth in 5 g/L isobutanol compared to parent[29]. However, n-butanol evolved *E. coli* strains showed decreased U/S, but still were able to increase the growth rate in n-butanol[30]. So while an increased tolerance to the hydrophobic compound

is common for the above examples, the mechanism with respect to the lipid profile changes can vary between individual evolved strains.

In contrast to the above *E. coli* findings, evolved *Clostridium* species consistently produce more saturated acyl chains compared to parent strains under solvent stress. A solvent evolved *C. beijerinckii* strain was shown to have nearly abolished production of unsaturated fatty acids and increased n-butanol production [31], and an ethanol-adapted strain of *C. thermocellum* had a fatty acid profile with a longer average acyl chain length, but no change in branching was observed[25].

Systems-biology analysis confirms that energy metabolism is disrupted by toxic-chemical induced membrane stress

It has been known that membrane disruption negatively affects the proton gradient and electrochemical potential, and thus the membrane proton motive force[2]. Disrupted proton and ion gradients lead to energetic problems, as the proton motive force generates ATP in aerobic respiration and a minimum adenylate energy charge (a metric of cellular energy availability) is required for viability[32]. Ion gradients, also affected by membrane disruption, are used in active transport processes. Systems biology approaches have enabled greater insight into the genetic, transcriptomic, and metabolomic responses to exogenous stress and the likely mechanisms of tolerance to membrane-disrupting chemicals.

It has been shown through metabolic flux analysis of *E. coli* under octanoic acid stress that carbon flux at the pyruvate node favors the NAD-independent PoxB-catalyzed pyruvate oxidation reaction to acetate, and a ubiquinol ($\Delta_r G^\circ = -150$ kJ/mol) instead of the NAD-dependent pyruvate decarboxylation reaction (*aceEF-lpd*) leading to acetyl-CoA, carbon dioxide, and NADH ($\Delta_r G^\circ = -35$ kJ/mol)[33,34]. The former reaction is more thermodynamically favorable compared to the latter as it produces acetate instead of acetyl-CoA. Acetyl-CoA can enter central metabolism directly or be used to produce an ATP in its conversion to acetate. Consistent with these findings, the transcriptional regulator PdhR, which negatively controls the pyruvate dehydrogenase complex genes (*aceEF-lpd*) as well as genes in the terminal electron transport chain (*ndh* and *cyoABCDE*)[35], has been shown to be upregulated in *E. coli* undergoing acid stress[36]. Increased expression of PoxB, strictly regulated by RpoS, may relieve oxidative stress in *E. coli* by reducing flux through the pyruvate dehydrogenase complex, which avoids the production of reactive oxygen species by NADH dehydrogenase[37]. This diverted flux reduces the carbon flow through the TCA cycle and reduces NADH production destined for the electron transport chain, increasing relative acetate production and greatly

reducing biomass[34]. In acetate genomic library selections, increased biomass formation was associated with overexpression of regulated genes in the TCA cycle and biomass production pathways (e.g. amino acid and pyrimidine synthesis)[38].

Isobutanol stress in *E. coli* has been shown to disrupt the function of the membrane-embedded electron carrier quinone, which subsequently disrupts cellular respiration and is postulated to be the source of a regulatory response cascade in *E. coli*[39]. Likewise, changes in expression of the transcription factors ArcA, PdhR, and Fur led to the downregulation of the *nuoABCEFGHIKL* operon (NADH dehydrogenase I), the *cyoABCDE* operon (cytochrome *o* oxidase), and genes associated with the TCA cycle and upregulated the *ndh* gene (NADH dehydrogenase II)[39]. Wild type *E. coli* under n-butanol stress showed an upregulation of genes related to respiratory functions (*nuo*, *sdh*, and *cyo* operons) and the TCA cycle[40]. Overexpression of membrane-related genes *nuoI* and *pgsA* (cardiolipin biosynthesis) increased n-butanol tolerance in *E. coli*[41]. Transcriptomic analysis of the ethanol tolerant *E. coli* strain LY01, versus its parent KO11 strain, has shown that LY01 has increased expression of the *cyo* operon and *ndh* gene while the *nuo* operon was down regulated[42]. Genes associated with the aerobic TCA cycle were also generally upregulated in the LY01 strain; ArcA, part of the quinone-dependent ArcAB two-component transcription factor system was expressed higher in the parent KO11 than in the ethanol tolerant LY01[42]. These studies collectively show the importance of the cellular energetics as a source of toxicity and tolerance in cells under membrane stress.

Engineering of the core lipid metabolism alters the fatty acid profile for engineering tolerance

With insight from natural mechanisms of tolerance, including information from naturally solvent tolerant bacteria and evolved mutant strains, engineering the lipid content of the cellular membrane by overexpression or deletion of core lipid synthesis genes has been tested in a variety of contexts.

Overexpression of the β -hydroxydecanoyl thio-ester dehydrase/isomerase (FabA), which converts 3-hydroxydecanoyl-acyl carrier protein (ACP) to *cis*-2-decanoyl-ACP, increases the relative amount of saturated fatty acids and increases ethanol tolerance (Fig. 1)[43]. This agrees with results with an *E. coli* genome library-based method which showed *fabA* was selected after ethanol selection[44]. Conversely, expression of the fatty acid desaturase gene from *B. subtilis*, *des*, decreased the relative amount of saturation and decreased ethanol tolerance[43]. Overexpression of *fabD*, important as a rate-limiting step in fatty acid biosynthesis, improved growth in n-butanol stress in *E. coli* by increasing the average acyl chain length and increasing

U/S[45]. Deletion of the fatty acid degradation regulator, FadR, which induces expression of fatty acid biosynthesis genes (*accABCD*, *fabH*, *fabB*, and *fabA*, Fig. 1), increased the saturated fatty acid content compared to the parent and increased hexane tolerance in *E. coli*[46].

Cyclopropane fatty acids are important in membrane homeostasis

Cyclopropane fatty acids (CFAs) are a substantial portion of the fatty acid content of a variety of bacteria, including *E. coli*, and are important for membrane lipid homeostasis[19]. CFAs are important to acid resistance in *E. coli*[47]. CFAs have been implicated in solvent tolerance in *P. putida* where *cfa* (encoding CFA synthase) is under the control of the stress-specific RpoS sigma factor[48,49]. The cyclopropanation reaction occurs within the membrane on existing unsaturated fatty acids by the addition of a methylene group from an S-adenosyl-L-methionine (Fig. 1). CFA synthesis does not require ATP or *de novo* fatty- acid synthesis, making it an attractive target in engineering applications.

Upregulation in *cfa* was observed in *E. coli* under octanoic acid, isobutanol and ethanol stress[39,50]. Expression of the *cfa* gene from a butanol tolerant *Enterococcus faecalis*-related strain in *E. coli* resulted in a significant increase in the CFAs and the growth rate of the recombinant strain in both isobutanol and n-butanol[23]. However, either deletion or overexpression of *cfa* was found to be inhibitory to growth compared to the wild type strain on various octanoic acid concentrations, and both had profound effects on the membrane lipid profile: overexpression of *cfa* nearly abolished UFAs, and its deletion prevented formation of CFAs [50]. Significant downregulation of *cfa* was observed in *Lactobacillus brevis* under n-butanol stress which reduced the CFA content[51].

Transcriptional analysis of several n-butanol-tolerant *E. coli* mutants derived from a combination of n-butanol selection and protoplast-fusion based genome shuffling showed significant upregulation of *cfa*, and *cfa* overexpression increased the growth rate by 14% in 0.5% (v/v) n-butanol[30]. However, a similar study of an isobutanol-tolerant *E. coli* mutant (EcNR1 G3.2) showed a significant downregulation of *cfa* and a corresponding reduction in CFA content in the mutant[29]. In contrast, *cfa* in *C. acetobutylicum* was shown to be upregulated under acid stress or during acidogenesis, but only upregulated at the beginning of n-butanol stress or solventogenesis[52,53]. Overexpression of *cfa* in *C. acetobutylicum* increased both acid and solvent tolerance, but reduced solvent production, perhaps because a modified membrane perturbed the regulation of solvent production[54]. The differences observed with respect to overexpression of *cfa* points to subtleties in regulation that are required for proper maintenance

of membrane homeostasis. While the relationship between CFAs and chemical tolerance is not linear or fully elucidated at this time, it is clearly an area of interest for future work.

Trans-unsaturated fatty acids for modulating membrane density for enhanced tolerance

In some Gram⁻ strains, the periplasmic *cis-trans* isomerase (Cti) converts unsaturated fatty acids from the *cis* to the *trans* conformation as a short term mechanism to maintain membrane fluidity under solvent and temperature stress (Fig. 1)[55]. Cti, not native to *E. coli*, has been implicated in solvent tolerance by providing a rapid mechanism to increase phospholipid density in the membrane, as the *trans*-unsaturated fatty acid is more linear in form, akin to a SFA[56]. The *cis-trans* isomerase gene (*cti*) from *P. aeruginosa* was expressed in *E. coli* and shown to improve tolerance and increase production of octanoic acid by increasing the membrane rigidity[57]; subsequent investigation revealed Cti expression also conferred tolerance to a range of physiological stress conditions including low pH, osmotic pressure, high temperature, and various chemical stressors such as short chain fatty acids, alcohols, organic acids, and aromatic compounds[57].

Phospholipid headgroups as area for continued exploration

Engineering specific headgroups of glycolipids is a potential exploration area for engineering tolerance as polar headgroups have been shown to be of importance in maintaining membrane homeostasis[58]. For example, *C. acetobutylicum* under octanol stress modifies the polar headgroups of lipids to stabilize the bilayer by increasing the presence of the charged ethanolamine-phosphate group to certain diglycerides[59]. In a computer simulation, lipid bilayers with phosphatidylcholine (PC, common in eukaryotes, rare in prokaryotes) headgroups were more susceptible to ethanol permeation compared to those with phosphatidylserine (PS) or phosphatidylethanolamine (PE)[60]. Conversely, knocking out PC synthesis in *Acetobacter aceti*, an acetic-acid tolerant strain, resulted in an acetic acid sensitive strain while ethanol tolerance was unaffected[61]. Much is left to be understood in how the choice of the polar headgroups could be engineered for improved tolerance and production in metabolic engineering applications.

Beyond the membrane: the cell-wall components, peptidoglycan and lipopolysaccharides act as outer defense barriers

It is common for strains evolved for tolerance to solvents or alcohols to have modifications to the LPS or peptidoglycan, as strengthening these structures can protect the cell from harm.

An evolved n-butanol-tolerant *E. coli* mutant had differences in expression in many lipopolysaccharide and peptidoglycan biosynthesis genes[30]. Significant changes in expression (mostly increases) of the outer membrane enterobacterial common antigen (ECA) gene cluster (*rffH*, *rffA*, *rffM*), lipopolysaccharide synthesis and transport (*lpcA*, *glmM*, *lpxK*, *lpxL*, *lptA*), and peptidoglycan biosynthesis (*glmM*, *murE*, *murF*, *amiB*) were all seen in the n-butanol tolerant mutants[30]. An isobutanol-tolerant mutant of *E. coli* was found to have an inactivating insertion in the *yhbJ* gene, a negative regulator of the *glmS* gene[62]. Overexpression of the *glmS* gene may lead to overproduction of glucosamine-6-phosphate, which is a precursor in both peptidoglycan and LPS synthesis (Fig. 2). The isobutanol-tolerant *E. coli* strain EcNR1 G3.2 had multiple mutations in lipopolysaccharide (LPS) related genes (i.e. *fepE*, *yjgQ*) and upregulation in the *rfa/waa* gene cluster, which encodes for LPS biosynthesis (Fig. 2b)[29]. An overall theme of upregulation of genes and gene clusters related to LPS and peptidoglycan biosynthesis is observed in these n-butanol and isobutanol tolerant mutants.

Genomic library selections on various growth inhibitory compounds consistently result in the identification of genes involved in peptidoglycan and LPS biosynthesis. The *lpcA* gene, coding the first committed step in the LPS inner core precursor pathway (Fig. 2b), has been selected for using the scalar analysis of library enrichments (SCALES) method against acetate, furfural, and ethanol[38,63,64]. SCALES selection against furfural, a hydrophobic lignocellulosic hydrolysate byproduct known to cause membrane disruption[6], also showed that high copy number of genes related to synthesis of the outer membrane glycolipid ECA (enterobacterial common antigen, *rffA*, *rffC*, *rffD*, *rffE*, *rffG*, *wzxE*) and high copy number of the division and cell-wall synthesis (dcw) gene cluster (*murEF-mraY-murD-ftsW-murGC-ddlB-ftsQ*, Fig 2.) resulted in increased fitness[64]. Under ethanol selective pressure, the 2nd highest ranked gene in a SCALES selection was the peptidoglycan maturation gene *mrdA*[44], and in an acetate SCALES selection the highest ranking gene was *murC*[38]. Goodarzi *et al.* showed that overexpression of peptidoglycan biosynthesis genes (*murB*, *murE*, *murF*, *murD*, *dacA*) and a deletion of the peptidoglycan hydrolyzing gene *slt* increased ethanol tolerance[65], which corresponds well with Nicolaou *et al.*'s library screening strategy which identified both the *murEF-mraY-murD* portion of the dcw cluster in a fosmid and the *murA-ibaG-mlaBCDEF* gene cluster as beneficial for ethanol tolerance [66]. It is interesting to note that many peptidoglycan genes found in the genomic library selections are those that are repressed by PdhR, possibly indicating a regulatory mechanism at work. A strategy for exploring heterologous DNA to find genetic elements conferring product tolerance in *E. coli* has been used to positive effect. A library *Lactobacillus plantarum* genes randomly inserted into the *E. coli* KO11 chromosome

revealed that expression of the heterologous *murA2* gene increases ethanol production and tolerance[67].

Unexpectedly, transcriptomic data show reduced peptidoglycan biosynthesis gene expression in ethanol tolerant LYO1 compared to KO11[42]. Diaminopimelate synthesis genes (*lysC*, *asd*, *dapB*, and *dapD*) were downregulated in LYO1 strain, while *lysA* was upregulated (Fig. 2a)[42]. Transcriptional increases were seen in *mepA* (murein endopeptidase), and *kdsA* (CMP-Kdo production, Fig. 2b) while decreases were seen in *lpp* (major lipoprotein tying the inner membrane to the peptidoglycan, Box 1) and *murF* in the LYO1 compared to KO11[42]. This stands in contrast to the butanol-tolerant mutants and ethanol genomic library selections discussed above where cell wall synthesis genes were found to be upregulated or have increased fitness.

Recommendations for future direction: a call for a synthetic membrane and cell-wall project

Analysis of the current literature supports the crucial role of cell membrane and wall in combating chemical toxicity and that innate and engineered changes in their composition suggest them as important engineering targets. While so far only one or a few genes affecting membrane and cell-wall biosynthesis have been engineered, large-scale engineering of these critical cellular components will likely provide better solutions for combating chemical toxicity. Synthetic biology has reached a level of maturity that enables a large-scale engineering of core biosynthetic programs (Figs. 1 & 2) and the engagement of non-native genes and intermediates that could give rise to robust, functional membrane structures. One could envision the addition of chemical intermediates, whether externally provided or via engineered biosynthesis, that would generate altered membrane and cell-wall structures, from *trans*-unsaturated fatty acids, novel or enhanced phospholipid headgroups, to dramatically altered core lipid metabolism, enabling extensive changes in the fatty-acid profile. The breadth of the natural repertoire engaged by microbes to deal with chemical toxicity remains largely unexplored, and could guide synthetic biology driven membrane engineering. For example, although it is well known that several *Lactobacilli* possess unusually high tolerance to solvents and other chemicals[2], the precise nature of it remains a mystery; surely membrane and cell-wall composition are part of the answer for the extraordinary tolerance of these small-genome microbes. Another case study that could guide engineered approaches is that of the alcohol tolerant *Zymomonas mobilis*. As reviewed[2], its membranes contain over 75% vaccenic acid (18:1) in polar lipids, while its cytoplasmic membrane contains hopanoids that apparently stabilize the phospholipid bilayer to resist solvent fluidization. These are but a few of the likely large number of unusual membrane

and cell-wall compositions that have evolved in nature to handle chemical toxicity and such capabilities could be explored through modern metagenomic tools and screens[68,69]. Thus, we call for both such naturally inspired and knowledge-driven *ab initio* design of cell membranes and walls that would exceed the best of the most tolerant organisms known, e.g., species of *Pseudomonas*, *Zymomonas*, *Lactobacillus*, *Rhodococcus*, *Deinococcus*, and *Nocardioides*[2]. This ‘*synthome*’ project could be undertaken through a coordinated, multidisciplinary and multi-team strategy that would aim to holistically attack and resolve the microbial toxicity problem that remains core to the success of microbial processes for the production of chemicals and fuels.

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Box

The bacterial membrane and cell wall of peptidoglycan and lipopolysaccharide (for Gram⁻) provide a first line of defense against exogenous threats to the cell. The cytoplasmic membrane consists of phospholipids from the fatty acid biosynthesis pathway (Fig. 1) with varying degrees of *cis*-unsaturation which can be modified to cyclopropanated fatty acids or *trans*-unsaturated fatty acids. Peptidoglycan, or murein, is a polymer network comprising amino acids and sugars formed in lipid II biosynthesis (Fig. 2a) that is bound to the membrane by lipoprotein. The peptidoglycan layer is thick in Gram⁺ bacteria (>20 nm) compared to Gram⁻ bacteria (~7 nm), where it is situated in the periplasm. Lipopolysaccharide (LPS) is the outermost feature of Gram⁻ bacteria and consists of the lipid A from the outer membrane and a polysaccharide chain which varies from strain to strain which forms a monolayer on the cell surface (Fig. 2b). While these peptidoglycan and LPS related genes are often implicated in tolerance to various stressors, engineering these structures is an area ripe for exploration.

Parent/ Control Strain	Evolved/ Engineered strain	Condition	U/S Ratio	CFA	Chain Length	Growth	Production	Reference
<i>E. coli</i> ML115 (fadD poxBackA- pta:cmR)	LAR1 (evolved on octanoic acid)	No Stress	Up	Up	ND	-	Increased C8	Royce (2015)[28]
		30 mM octanoic acid, pH 7.0	Up	Slightly Up	Up	Increase	N/A	
		430 mM ethanol	ND	Up	ND	-		
		30 degC	Down	ND	ND	-		
		42 degC	Up	Up	Up	-		
<i>E. coli</i> BW25113	MY4 (evolved on n-butanol)	Not stated	Down	Up	-	Increase	N/A	Reyes (2012)[30]
	MG6 (evolved on n-butanol)	Not stated	Down	Up	-	Increase	N/A	
<i>E. coli</i> EcNR1	G3.2 (evolved on isobutanol)	0.5 %w/v isobutanol (67.5 mM)	Up	Down	-	Increase	N/A	Minty (2011)[29]
<i>C. beijerinckii</i> ATCC 10132	‘solvent adapted’ <i>Cbei</i>	Not stated	Down	-	-	Increase	Increased n- butanol	Isar (2012)[31]
<i>E. coli</i> MG1655	MG1655 Δcfa	No Stress	ND	Zero	ND	Decrease	N/A	Royce (2014)[50]
		10 mM octanoic acid	Up	Zero	ND	ND		
		20 mM octanoic acid	ND	Zero	ND	ND		
	MG1655+pCA- <i>cfa</i>	No Stress	ND	Up	ND	Decrease		
		10 mM octanoic acid	Down	Up	Down	Decrease		
		20 mM octanoic acid	Down	Up	Down	Decrease		
<i>E. coli</i> M1- Control (MG1655, ldhA: FRT-Cat-FRT)	M1-12-Pa_cti (plasmid based <i>P.aeruginosa cti</i>)	10 mM octanoic acid, pH 7.0	ND	-	ND	Increase	Increased C8	Tan (2016)[57]
		343 mM ethanol	-	-	-	Decrease	N/A	
		66 mM n-butanol	-	-	-	Increase		
		66 mM isobutanol	-	-	-	ND		
		42 degC	-	-	-	Increase		
<i>E. coli</i> DH5 α + pBR322	DH5 α + pBR-des (<i>B. subtilis</i> fatty acid desaturase)	No Stress	Up	Down	Down	ND	N/A	Luo (2009)[43]
		3% v/v Ethanol (514 mM)	Up	Down	Up	Decrease		
	DH5 α + pBR-fabA (<i>fabA</i>)	No Stress	Down	Down	Down	ND		
		3% v/v Ethanol (514 mM)	Down	Slightly Up	Down	Increase		
<i>E. coli</i> MDB5	MDB5 + pAD3 (<i>fabD</i>)	1% n-butanol (135 mM)	Up	-	Up	Up	N/A	Bui (2015)[45]
<i>E. coli</i> RLo8 (K- 12 Δ fadD Δ araBAD)	RLo8 Δ <i>fabR</i>	No Stress	Up	-	-	-	N/A	Lennen (2013)[70]
	RLo8 + p <i>fabR</i>	No Stress	Down	-	-	-	N/A	
<i>E. coli</i> BW25113	JW1176 (Δ <i>fadR</i>)	No Stress	Down	Down	Down	-	N/A	Oh (2012)[46]

Parent/ Control Strain	Evolved/ Engineered strain	Condition	U/S Ratio	CFA	Chain Length	Growth	Production	Reference
		10% v/v <i>n</i> -hexane (760 mM)	Down	Up	Down	Increase	N/A	

Table 1. Differences in adaptive responses of evolved or engineered strains compared to the response of the parent strain in the given condition. U/S = unsaturated to saturated ratio. ND = No Difference. - = no data given. N/A = Not Applicable.

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Figure captions

Figure 1. Type II fatty biosynthesis pathway map and unsaturated fatty acid modifications with *E. coli* genes associated with the reactions shown. *accABCD* encodes acetyl-CoA carboxyltransferase, *fabD* malonyl-CoA-ACP transacylase, *fabH* β -ketoacyl-ACP synthase III (KASIII), *fabG* 3-oxoacyl-[acp] reductase, *fabZ* 3-hydroxy-acyl-[acp] dehydratase, *fabI* enoyl-[acp] reductase, *fabB* KASI, *fabA* β -hydroxyacyl-[acp] dehydratase/isomerase, *fabF* KASII, *plsB* glycerol-3-phosphate acyltransferase, *plsC* 1-acylglycerol-3-phosphate O-acyltransferase, *cfa* cyclopropane fatty acyl phospholipid synthase, and *cti* cis-trans isomerase. Green arrows represent gene activation and red lines represent repression. 'R' represents any phospholipid head group, [acp] acyl carrier protein, SAM S-adenosyl-L-methionine, and S-adenosyl-L-homocysteine. Repression by FabR is activated by unsaturated fatty acids.

Figure 2. Cell wall biosynthesis pathways. (A) Peptidoglycan precursor lipid II biosynthesis with *E. coli* genes associated with reactions. *pgi* encodes phosphoglucose isomerase, *glmS* L-glutamine:D-fructose-6-phosphate aminotransferase, *glmM* phosphoglucosamine mutase, *glmU* N-acetylglucosamine-1-phosphate uridyltransferase, *murA* UDP-N-acetylglucosamine enolpyruvoyl transferase, *murB* UDP-N-acetylenolpyruvoylglucosamine reductase, *murC* UDP-N-acetylmuramate-alanine ligase, *murI* glutamate racemase, *murD* UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase, *murE* UDP-N-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase, *dadX/alr* alanine racemase, *ddl* D-alanine-D-alanine ligase, *murF* UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase (final cytoplasmic step), *mraY* phospho-N-acetylmuramoyl-pentapeptide transferase, *murG* N-acetylglucosaminyl transferase, *lysC* aspartate kinase, *asd* aspartate semialdehyde dehydrogenase, *dapA* dihydrodipicolinate synthase, *dapB* tetrahydrodipicolinate succinylase, *serC/argD* N-succinyldiaminopimelate aminotransferase, *dapE* N-succinyl-L-diaminopimelate desuccinylase, *dapF* diaminopimelate epimerase, and *lysA* diaminopimelate decarboxylase. Red lines indicate repression by PdhR or lysine. (B) Lipopolysaccharide (LPS) lipid A-core biosynthesis with genes associated with reactions. *lpxA* encodes UDP-N-acetylglucosamine acyltransferase, *lpxC* UDP-3-O-acyl-N-acetylglucosamine deacetylase, *lpxD* UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acetyltransferase, *lpxH* UDP-2,3-diacylglucosamine hydrolase, *lpxB* lipid A disaccharide synthase, *lpxK* tetraacyldisaccharide 4'-kinase, *gutQ/kdsD* D-arabinose 5-phosphate isomerase, *kdsA* KDO-8P synthase, *kdsC* KDO-8P phosphatase, *kdsB* KDO cytidyltransferase, *waaA* KDO transferase, *lpxL/lpxP* an acyltransferase, *lpxM* myristoyl-

[*acp*] acyltransferase, *lpcA* D-sedoheptulose 7-phosphate isomerase, *rfaE* heptose phosphate kinase/adenyltransferase, *gmhB* heptose bisphosphate phosphatase, *waaC* ADP-heptose:LPS heptosyltransferase I (HepI), *waaF* HepII, *waaG* LPS glucosyltransferase I, *waaP* LPS core heptose kinase I (LCHK I), *waaQ* HepIII, *waaY* LCHK II, *waaB* LPS galactosyltransferase, *waaO* LPS glucosyltransferase II, *waaJ* LPS glucosyltransferase III, and *waaU* HepIV. Red lines indicate repression by PdhR.

Figure 1

Figure 2