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Genetic Determinants for *n*-Butanol Tolerance in Evolved *Escherichia coli* Mutants: Cross Adaptation and Antagonistic Pleiotropy between *n*-Butanol and Other Stressors

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Cross-tolerance and antagonistic pleiotropy have been observed between different complex phenotypes in microbial systems. These relationships between adaptive landscapes are important for the design of industrially relevant strains, which are generally subjected to multiple stressors. In our previous work, we evolved *Escherichia coli* for enhanced tolerance to the biofuel *n*-butanol and discovered a molecular mechanism of *n*-butanol tolerance that also conferred tolerance to the cationic antimicrobial peptide polymyxin B in one specific lineage (green fluorescent protein [GFP] labeled) in the evolved population. In this work, we aim to identify additional mechanisms of *n*-butanol tolerance in an independent lineage (yellow fluorescent protein [YFP] labeled) from the same evolved population and to further explore potential cross-tolerance and antagonistic pleiotropy between *n*-butanol tolerance and other industrially relevant stressors. Analysis of the transcriptome data of the YFP-labeled mutants allowed us to discover additional membrane-related and osmotic stress-related genes that confer *n*-butanol tolerance in *E. coli*. Interestingly, the *n*-butanol resistance mechanisms conferred by the membrane-related genes appear to be specific to *n*-butanol and are in many cases antagonistic with isobutanol and ethanol. Furthermore, the YFP-labeled mutants showed cross-tolerance between *n*-butanol and osmotic stress, while the GFP-labeled mutants showed antagonistic pleiotropy between *n*-butanol and osmotic stress tolerance.

Naturally produced by *Clostridium* species, *n*-butanol is an industrial intermediate chemical, a solvent, and a potential biofuel. Several nonnative microbial systems have been engineered for its production, including *Escherichia coli* (1), *Lactobacillus brevis* (2), *Pseudomonas putida* (3), *Bacillus subtilis* (3), and *Saccharomyces cerevisiae* (4). However, this solvent is highly toxic to microorganisms, imposing a limit on the productivity of bio-based production and leading to the development of simultaneous fermentation and separation techniques to mitigate the toxic effects of the biofuel (5) and efforts to identify the genetic determinants and molecular mechanisms associated with *n*-butanol tolerance for reverse engineering of more robust strains (6–12). Prior strain engineering efforts include overexpression of GroESL in *Clostridium acetobutylicum* (resulting in a 50% improvement in total growth in 0.75% [vol/vol] *n*-butanol) (12) and recently in *E. coli* (resulting in a 2.8-fold increase in total growth after 48 h in 0.75% [vol/vol] *n*-butanol) (11) and overexpression of gene CAC1869 in *C. acetobutylicum* (resulting in an 81% increase in total growth after 12 h) (13). Since *n*-butanol tolerance is a complex phenotype (6, 13) and the production environment involves multiple stressors, additional knowledge on the genetic determinants and molecular mechanisms involved and their effects under different stress conditions is essential for future strain engineering efforts.

We previously reported the use of an adaptive laboratory evolution-based method called visualizing evolution in real time (VERT) to study *n*-butanol tolerance in *E. coli* (7). Using a two-color VERT system (with green fluorescent protein [GFP]-labeled and yellow fluorescent protein [YFP]-labeled cells, allowing the tracking of independent lineages), we isolated several *n*-butanol-tolerant adaptive mutants throughout the evolution and used whole-genome transcriptome profiling and resequencing analyses to identify the underlying *n*-butanol tolerance mechanisms. A reduced activity of the ferric uptake regulator Fur, leading to increased siderophore biosynthesis and transport, which ultimately led to membrane modifications, was identified to be a likely mechanism of enhanced *n*-butanol tolerance. The deactivation of Fur also led to cross-tolerance between *n*-butanol and the cationic antimicrobial peptide polymyxin B. However, this tolerance mechanism was observed only in mutants from the GFP-labeled subpopulation and not in the YFP-labeled subpopulation, suggesting a different route(s) of adaptation in the YFP-labeled adaptive mutants. In this study, we aim to identify the mechanisms of *n*-butanol tolerance in the YFP-labeled mutants and any additional cross-resistance and/or antagonistic pleiotropy between *n*-butanol and other stressors in the isolated adaptive mutants. Detailed analysis of the transcriptome profiles of the yellow-labeled subpopulation under *n*-butanol stress was performed to determine additional genetic determinants involved in tolerance to the solvent. Phenotypic analyses revealed divergent relative fitness profiles in different stressors between the two different lineages. Several genes related to membrane transporters and cardiolipin biosynthesis, an important component of bacterial membranes, were determined to be involved in resistance to *n*-butanol exclusively among the solvents tested.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. The *E. coli* K-12 strain BW25113 [F$^-$ Δ(*araD-araB*)567 Δ*lacZ4787*(::*rrnB-3*) lambda$^-$ *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514*] obtained from the Coli Genetic Stock Center (CGSC) was used in this study. Plasmids isolated from clones in the ASKA(−) collection (14) were used for the overexpression studies in BW25113.

Growth condition and maintenance. *E. coli* strains were routinely cultured aerobically in liquid Luria-Bertani (LB) medium at 220 rpm and 37°C and on agar-solidified LB at 37°C. When required, the medium was supplemented with 30 μg/ml of chloramphenicol. Frozen stocks were prepared from cultures grown overnight and were stored in 17.5% glycerol at −80°C. Cells from a single colony were used to inoculate liquid cultures. Growth curves were carried out in M9 minimal medium supplemented with 5 g/liter of glucose, 0.01% (wt/vol) thiamine, and the appropriate antibiotic when required.

DNA isolation and transformation. Isolation of plasmid DNA from *E. coli* was performed using the Zyppy plasmid miniprep kit (Zymo). Electroporation was used for all *E. coli* transformations.

Prescreening of potential *n*-butanol tolerance-conferring genes. Strains harboring the genes to be prescreened were cultured in M9 minimal medium (5 g/liter glucose) and incubated overnight at 37°C to be used as the inoculum. The next day, 100-μl cultures were prepared in 96-well microtiter plates for growth kinetic analysis in the absence and presence of 0.8% (vol/vol) *n*-butanol in M9 medium (at this concentration of *n*-butanol, the growth rate of the parental strain was inhibited by more than 50%) at 37°C, using an Infinite M200 microplate reader (TECAN). Four technical replicates were obtained per sample in the prescreen. The growth kinetic parameter *s* described below was calculated. Statistical significance was assessed using Student's *t* test analysis using a *P* value cutoff of 0.05.

Calculation of growth kinetic parameters. Growth kinetic parameters, including percentage of inhibition, relative fitness coefficient (*s*), and relative increase in fitness (RIF), were calculated using equations 1, 2, and 3, respectively. These parameters were calculated using the measured maximum specific growth rate (μ_i) of each strain (*i*).

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{\mu_{\text{clone @ stressful condition}}}{\mu_{\text{clone in absence of stressor}}}\right)\right] \times 100\% \quad (1)$$

$$s\,(\%) = \left[\left(\frac{\mu_{\text{clone @ stressful condition}}}{\mu_{\text{reference strain @ stressful condition}}}\right) - 1\right] \times 100\% \quad (2)$$

$$\text{RIF (\%)} = \left[1 - \left(\frac{\text{inhibition}_{\text{clone @ stressful condition}}}{\text{Inhibition}_{\text{reference strain @ stressful condition}}}\right)\right] \times 100\% \quad (3)$$

The ratio between the specific growth rates of the strain of interest relative to the reference strain under each stress condition was determined using the relative fitness coefficient *s* (equation 2). The appropriate reference strains were used for each calculation. For overexpression studies, the reference strain is the wild-type strain harboring the empty vector. For the phenotypic analysis of the isolated mutants, the ancestral strain expressing the corresponding fluorescent protein was used. RIF is a parameter calculated to normalize the relative fitness of the overexpression strain in the presence of the stressor against any fitness defects/advantage exhibited by the strain in the absence of the stressor. Positive values of RIF represent a net increase in growth rates in the presence of the stressor. Student's *t* test analysis ($P < 0.05$) was used to assess significance of the aforementioned calculated kinetic parameters.

Detailed phenotypic analysis of selected *n*-butanol tolerance-conferring genes. Clones that showed a statistically significant increase in relative fitness in the presence of *n*-butanol from the prescreen were selected for secondary validation in batch cultures. Each strain was cultured in M9 minimal medium (5 g/liter glucose) and incubated overnight at 37°C to be used as the inoculum. The next day, a 5% (vol/vol) inoculum was used to seed a 30-ml culture in 250-ml closed-cap flasks. At least three biological replicates were used in each experiment. The stressors analyzed in this study were 0.8% (vol/vol) *n*-butanol, 1% (vol/vol) isobutanol, 4% (vol/vol) ethanol, 1.75 g/liter of acetate (as acetic acid), pH levels of 4.5 and 6.0 (titrated using HCl), and temperatures of 28°C and 42°C. Cultures were incubated at 37°C (except in temperature challenge experiments) with constant shaking at 220 rpm. Growth was monitored using spectrophotometry (optical density at 600 nm [OD_{600}]) until stationary phase was reached.

Osmotic stress experiments. Cells were grown in M9 minimal medium (5 g/liter glucose) overnight. To inoculate 5 ml culture in M9 minimal medium, 5% (vol/vol) culture was used and incubated at 37°C until an OD_{600} of ~0.6 was reached. Bacterial cultures were normalized to an OD_{600} of 0.3, and cells from 1 ml of the normalized culture were recovered by centrifugation. Quick aspiration of the supernatant was followed by resuspension of the cell pellet in 1 ml of 40% (wt/vol) glucose and incubated at 37°C for 2 h with constant shaking (220 rpm). After incubation, cells were pelleted and resuspended in 1 ml of M9 minimal medium and then diluted 1:100. A total of 100 μl of the diluted culture was plated on LB agar plates and incubated overnight at 37°C for colony counting. At least four biological replicas were used in this experiment. A nonstressed culture from the same population was used as the control to ensure consistency in dilution. The "relative increase in survival rate" was calculated as the ratio of the numbers of CFU, after the osmotic shock, between the mutant and the parental strains.

RESULTS

Phenotypic analyses of the isolated mutants in multiple stressors. In a previous study, we used VERT to isolate *E. coli* mutants with enhanced *n*-butanol tolerance (7). Two differentially labeled (with GFP or YFP), but otherwise isogenic, strains of *E. coli* were used for the evolutionary experiment in the presence of increasing concentrations of *n*-butanol, resulting in two different-colored subpopulations (independent lineages). Several mutants were isolated and characterized via phenotypic (relative fitness measurements), genotypic (whole-genome resequencing), and transcriptomic (gene expression microarrays) analyses. We reported differential mechanisms of enhanced tolerance between the two independent lineages. In the GFP-labeled mutants (MG2, MG5, and MG6), cross-tolerance between the cationic antibiotic peptide polymyxin B and *n*-butanol was identified. The resistance level to polymyxin B in these mutants increased gradually, with the mutants isolated later in the population exhibiting higher antibiotic resistance than earlier GFP-labeled isolates (7). However, this cross-resistance was not observed in the YFP-labeled mutants (MY1, MY2, MY3, and MY4), none of which exhibited changes in sensitivity to polymyxin B. These prior results led us to hypothesize that additional cross-tolerance and/or antagonistic pleiotropy between *n*-butanol and other stressors may be present in our isolated mutants.

To identify potential cross-tolerance and antagonistic pleiotropy between *n*-butanol and other industrially relevant stressors in the isolated mutants, we evaluated their relative fitness under different conditions. The stressors used include organic solvents (*n*-butanol, isobutanol, and ethanol), organic acid (acetate), acid stress (pH of 4.5 and 6.0), and temperature stress (42°C and 28°C). A detailed description of each condition is provided in Materials and Methods. The results are summarized in Fig. 1 and Table 1.

The two different lineages (GFP- and YFP-labeled mutants) exhibited notable differences in their responses to the applied stressors, as shown in Fig. 1. MY2 showed a 15% increase in relative fitness (*s*) compared to that of the ancestral strain in the presence of *n*-butanol but an increase in *s* of 30% in the absence of

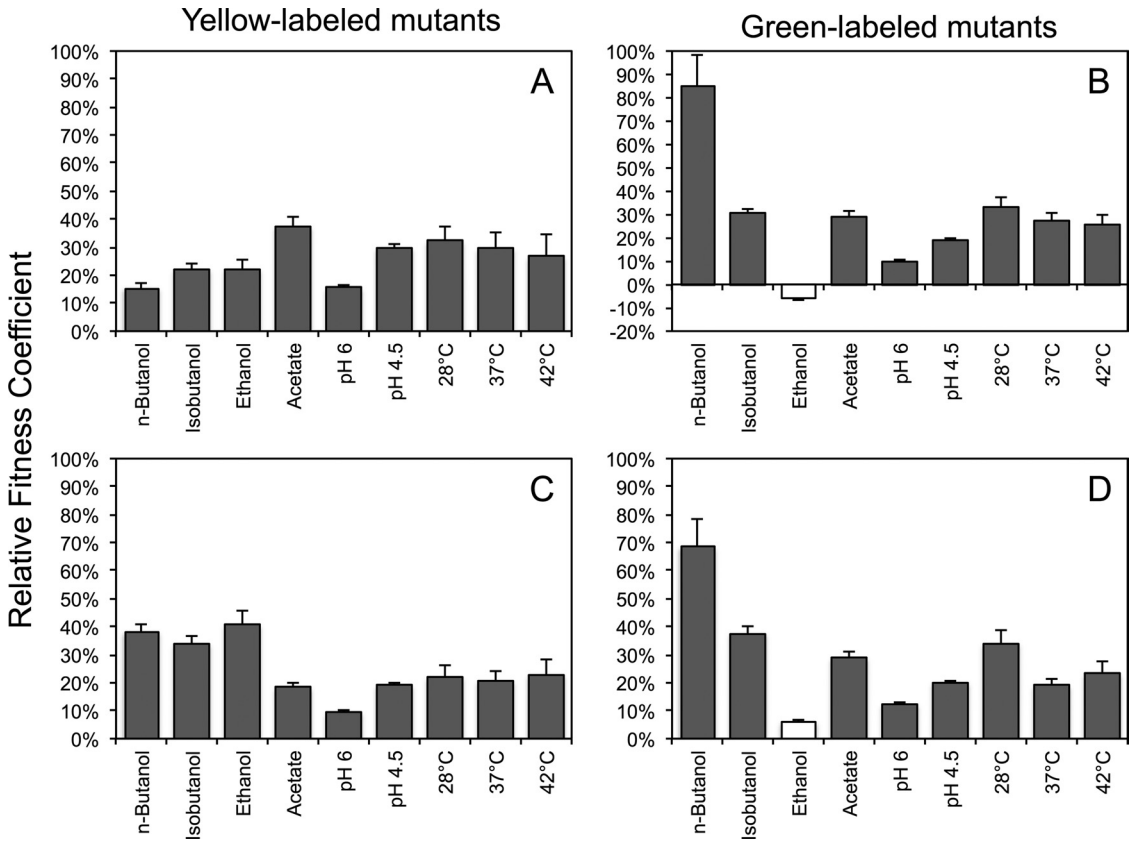

FIG 1 Cross-tolerance and antagonistic pleiotropy in the *n*-butanol-evolved mutants. (A) MY2; (B) MG5; (C) MY4; (D) MG6. Solid bars indicate statistically significant values, compared with the wild-type strain (Student's *t* test with a *P* value cutoff of 0.05).

n-butanol, indicating that the adaptive mechanisms were not specific for *n*-butanol tolerance, presumably via an increase in general nutrient utilization. Thus, the specific effects of each challenge on growth kinetics were normalized by calculating the RIF using equation 3, to determine if the increase in specific growth rate was the result of enhanced tolerance or due to a general increase in growth rate (as a result of increased nutrient utilization, energy levels in the cell, etc.). After normalization, the majority of the mutants exhibited negative values in RIF under many conditions, except in the presence of *n*-butanol, isobutanol, and acetate and at low temperatures (see Table 1), indicating that the isolated mutants do not have general tolerance to a wide range of stressors. Even among organic solvents, mutant MY4 (YFP marked) was the only mutant that showed an improvement in tolerance to all three organic solvents tested (*n*-butanol $s = 38\% \pm 3\%$, isobutanol $s = 34\% \pm 2\%$, and ethanol $s = 41\% \pm 6\%$). Interestingly, the GFP-labeled mutants did not exhibit an increase in relative fitness in the presence of ethanol, while the YFP-labeled mutants showed positive values of *s* under ethanol stress (Table 1).

The results presented in Table 1 demonstrated a potential antagonist pleiotropy between the molecular mechanisms involved in acid and *n*-butanol tolerance in the isolated mutants. In all the

TABLE 1 Growth kinetic parameters calculated for the isolated mutants under different stress conditions[a]

	S (%)				RIF (%)			
Stressor	MY2	MG5	MY4	MG6	MY2	MG5	MY4	MG6
n-Butanol	**15 ± 2**	**85 ± 13**	**38 ± 3**	**68 ± 10**	−48 ± 10	**216 ± 44**	**84 ± 15**	**261 ± 51**
Isobutanol	**22 ± 2**	**30 ± 2**	**34 ± 2**	**38 ± 3**	−26 ± 5	**13 ± 2**	**64 ± 11**	**99 ± 15**
Ethanol	**22 ± 3**	−6 ± 1	**41 ± 6**	6 ± 1	**−25 ± 6**	**−122 ± 23**	**96 ± 20**	**−68 ± 13**
Acetate	**37 ± 4**	**29 ± 3**	**18 ± 2**	**29 ± 2**	**26 ± 5**	8 ± 1	−12 ± 2	**52 ± 8**
pH 6.0	**15 ± 1**	**10 ± 1**	**9 ± 1**	**12 ± 1**	**−48 ± 9**	**−62 ± 9**	**−54 ± 9**	**−34 ± 5**
pH 4.5	**30 ± 1**	**19 ± 1**	**19 ± 1**	**20 ± 1**	0 ± 0	**−30 ± 4**	−8 ± 1	5 ± 1
$T = 28°C$	**35 ± 5**	**33 ± 4**	**19 ± 3**	**34 ± 5**	**19 ± 4**	**24 ± 4**	**−9 ± 2**	**80 ± 15**
$T = 37°C$	**30 ± 5**	**27 ± 4**	**21 ± 3**	**19 ± 3**	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$T = 42°C$	**27 ± 8**	**26 ± 5**	**22 ± 5**	**23 ± 4**	−10 ± 4	−5 ± 1	10 ± 3	**22 ± 5**

[a] The specific growth rates at $T = 37°C$ were used as references for RIF calculations. Statistically significant values are bolded. In the case of *s*, the bolded values indicate fitness coefficients significantly different from those of the wild-type strain (Student's *t* test with a *P* value cutoff of 0.05). For RIF, the bold values indicate measurements significantly different from 0.

TABLE 2 Summary of the list of genes that showed increased *n*-butanol tolerance when overexpressed in the ancestral strain BW25113[a]

Overexpressed gene	Gene ID	*s*		RIF		Function
		Average (%)	*P* value	Average (%)	*P* value	
sodB	b1656	24 ± 2	0.01	10 ± 1	0.00	Iron-superoxide dismutase
gcvH	b2904	23 ± 2	0.00	9 ± 1	0.00	Glycine cleavage system
hyfD	b2484	25 ± 2	0.00	10 ± 1	0.01	Hydrogenase
nuoI	b2281	22 ± 1	0.00	11 ± 1	0.00	NADH:ubiquinone oxidoreductase
pepB	b2523	40 ± 3	0.00	7 ± 1	0.00	Proteinase
treF	b3519	37 ± 3	0.00	7 ± 1	0.00	Trehalose biosynthesis
ygfO	b2882	35 ± 2	0.00	19 ± 1	0.00	Proton motive xanthine transporter
seta	b0070	24 ± 3	0.04	8 ± 1	0.01	Sugar efflux pump
mdtA	b2074	11 ± 1	0.00	5 ± 1	0.00	Drug resistance efflux pump
pgsA	b1912	15 ± 1	0.00	15 ± 1	0.00	Involved in cardiolipin biosynthesis

[a] The *s* and RIF values were determined in the presence of 0.8% (vol/vol) *n*-butanol.

mutants studied, RIF values calculated at pH 4.5 and 6.0 were either negative or nonsignificant (Table 1), suggesting a divergence in the tolerance levels under these two conditions. This antagonistic behavior between the two stresses has been documented previously in *E. coli* (6) and *L. brevis* (15).

Identifying genetic determinants underlying *n*-butanol tolerance in the YFP-labeled mutants. The phenotypic results demonstrated different molecular mechanisms of *n*-butanol tolerance between the two lineages from the evolution experiment. To determine the molecular mechanisms behind the differences in phenotypic profiles observed between the GFP-labeled and YFP-labeled mutants, we analyzed the transcriptome profiles in the YFP-labeled mutants to identify potential genetic determinants underlying *n*-butanol tolerance for further analysis. We focused our analysis on the top ~10% of the upregulated genes related to membrane processes, stress response, or global regulation from each of the YFP-labeled mutants (MY2, MY3, and MY4). A total of 46 genes were selected for further characterization by overexpressing each in the BW25113 parental strain.

Initially, a prescreen of the candidate genes was carried out in a high-throughput manner via growth kinetic analysis in the presence of 0.8% (vol/vol) *n*-butanol using a microplate reader. The genes that, when overexpressed, showed potential enhanced tolerance to *n*-butanol were further verified using batch cultures in close-capped flasks. The two kinetic parameters, *s* and RIF, were calculated to assess the improvement in *n*-butanol tolerance in the overexpression strains. Ten genes were found to have statistically significant effects on increasing *n*-butanol tolerance when overexpressed, and their functions include transporters, membrane components, and stress response (Table 2). Table S1 in the supplemental material includes the list of all the screened genes and their different kinetic parameters.

Membrane-associated genes. Modifications in the outer and cytoplasmic membranes are among the most common mechanisms for solvent tolerance in *E. coli*. We found several genes that encode membrane-associated proteins to be upregulated in the YFP-labeled mutants. The roles of several of these genes (*ygfO*, *setA*, *mdtA*, and *pgsA*) in enhancing *n*-butanol tolerance were confirmed via overexpression studies in wild-type *E. coli* (see the data in Table 2). The multidrug transporter ABC (*mdtABC*) operon, encoding the resistance-nodulation-cell division (RND) drug efflux system, is responsible for resistance against different compounds (16). Genes within the *mdt* operon have been previously found to be upregulated in *n*-butanol stress in *E. coli* (10). Several efflux pumps are known to be involved in solvent tolerance in bacteria, such as *srpABC* from *P. putida* in the export of octanol, hexane, and other hydrocarbons (17) and the *acrAB-tolC* pump in *E. coli* in tolerance to hexane, heptane, octane, and nonane (18), and several other heterologously expressed pumps in *E. coli* conferred tolerance to different solvents (19). However, no efflux pumps have been identified to be effective in exporting short-chain alcohols, such as *n*-butanol (19). The other membrane-related genes include the sugar transporter *setA*, the xanthine transporter *ygfO*, and a biosynthetic gene for the phospholipid cardiolipin, *pgsA*.

In order to determine whether the overexpression of the above-mentioned genes enhance general tolerance in the presence of growth inhibitors or exclusively in enhancing tolerance to *n*-butanol, their effects under different conditions (0.8% [vol/vol] *n*-butanol, 4% [vol/vol] ethanol, 1% [vol/vol] isobutanol, and 1.75 g/liter acetate) were assessed. The results are depicted in Fig. 2. Interestingly, the overexpression of *ygfO*, *mdtA*, and *pgsA* significantly decreased fitness when the strains were grown in the presence of isobutanol. In the case of *ygfO* overexpression, growth was completely inhibited in the presence of isobutanol, at least within the 12 h during which the experiment was conducted (Fig. 2A). No cross-tolerance between *n*-butanol and ethanol was observed when any of the genes were overexpressed. Overexpression of *ygfO* significantly decreased fitness in all the conditions tested except for *n*-butanol, suggesting the mechanism of *n*-butanol tolerance in this strain caused a broad-range antagonistic pleiotropy with other inhibitors. Cross-tolerance between acetate and *n*-butanol stress has been identified previously in *C. acetobutylicum* (3, 20) and thus was included as a test condition here. In the case of acetate, *setA* was the only gene that conferred enhanced tolerance when overexpressed.

Even though these membrane-related genes were identified based on the transcriptome data of the YFP-labeled mutants, the phenotypic profiles related to the overexpression of individual genes (Fig. 2) differ from those observed in mutants MY2 and MY4 (Fig. 1).

Osmotic stress. The transcriptome analyses revealed several genes known or potentially involved in osmoprotection (*gcvH*, *treF*, *setA*, and *pgsA*) to be perturbed in the YFP-labeled mutants but not in the GFP-labeled mutants. Overexpression of these genes in a wild-type strain was found to improve *n*-butanol tolerance, as shown in Table 2. Glycine and glycine-betaine (coded by members of the GcvA and BetI regulons) have been identified to

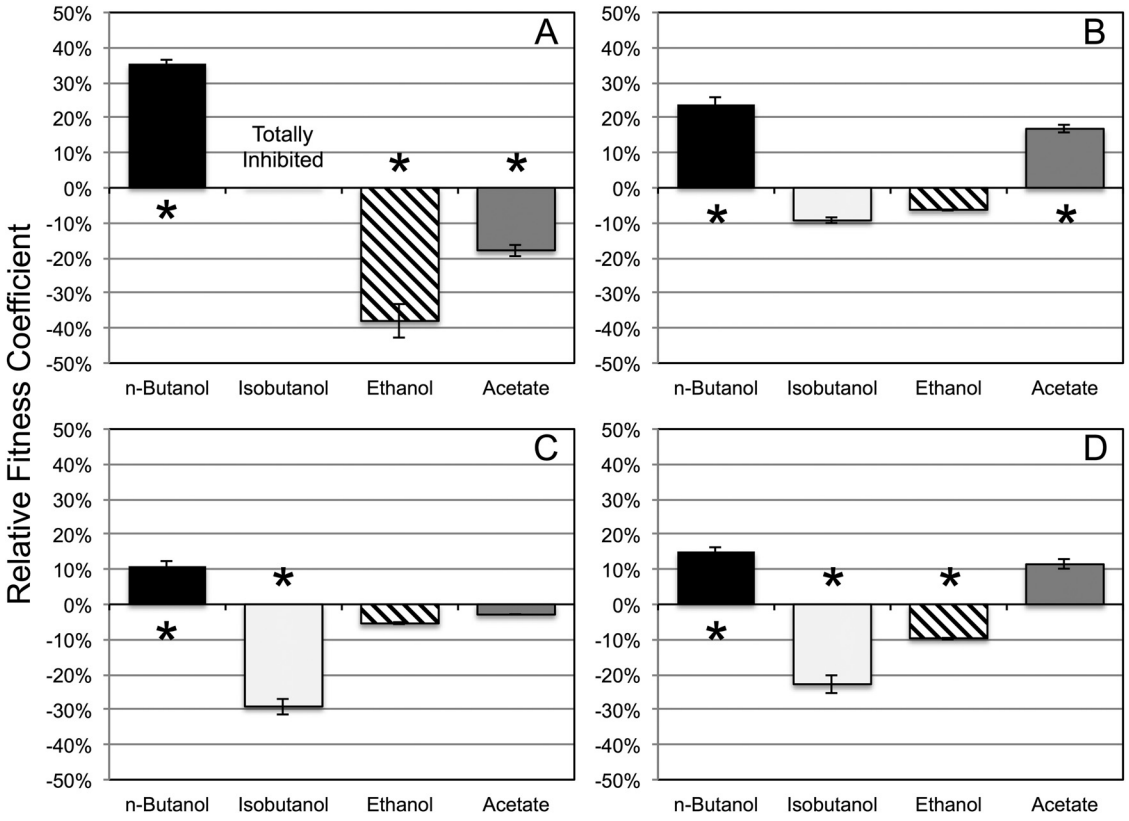

FIG 2 The relative fitness coefficient of overexpression of genes related with different transporters and membrane components. (A) *ygfO*; (B) *setA*; (C) *mdtA*; (D) *pgsA*. The asterisks indicate statistically significant values using Student's *t* test ($P < 0.05$). Growth was completely inhibited when *ygfO* was overexpressed in the presence of 1% (vol/vol) isobutanol.

contribute to ethanol tolerance in *E. coli*, possibly through higher production of osmolytes (3, 21). The biosynthesis of the disaccharide trehalose (produced by the cytoplasmic TreF and the periplasmic TreA) acts as an osmotic and stress protectant in *E. coli* (4, 22). This osmoprotectant action was of importance in the presence of different osmotic agents, such as inorganic salts and high concentrations of hexose sugars. SetA is an efflux pump capable of transporting different sugars, and the expression of *setA* was found to be increased under glucose/phosphate stress (5, 23), potentially alleviating the accumulation of nonmetabolized sugar phosphates. However, SetA also transports, albeit inefficiently, other substrates, such as the antibiotics kanamycin and neomycin, as well as glucosides and galactosides with alkyl or aryl substituents (6–12, 24). Since there are no sugar analogs in the medium, it is currently unclear whether the overexpression of *setA* contributes to osmoprotection in MY2 and MY4 mutants.

The gene *pgsA*, related to the biosynthesis of the phospholipid cardiolipin, was perturbed in MY2, suggesting a potential increase in cardiolipid biosynthesis in this mutant. Cardiolipin (CL) is a glycerophospholipid that is important in microbial cell membranes (12, 25). CL plays important roles in bacterial cell division (5, 11, 26), osmotic stress (13, 27), and essential function in the bioenergetics of the cell (6, 13, 28). In *E. coli*, *pgsA* encodes the committed step of CL biosynthesis. Overexpression of *pgsA* has been shown to modify cellular phospholipid composition, by increasing the concentrations of the acidic phospholipids phosphatidylglycerol and CL (7, 29).

Since several potentially osmoprotection-related genes were overexpressed in the evolved YFP-labeled mutants, and their overexpression was verified to be involved in *n*-butanol tolerance, we hypothesized that the YFP-labeled mutants are also more tolerant to osmotic stress. An osmotic shock experiment using high concentrations of glucose (40% [wt/vol]) as described in Materials and Methods was used. As shown in Fig. 3, statistically significant improvements in relative survival rates were seen in the three YFP-labeled mutants (244% for MY2, 499% for MY3, and 655% for MY4). Cross-tolerance between osmotic tolerance and solvent stress responses has been observed previously: cross-tolerance between ethanol and osmotic stress in *E. coli* (14, 22, 30) and *S. cerevisiae* (7, 31, 32) and between *n*-butanol and osmotic stress in *E. coli* (7, 33) and *Clostridium* (6, 34). Intriguingly, the GFP-labeled mutants show antagonistic pleiotropy between *n*-butanol and osmotic stress (relative survival rate of −65% for MG5 and −41% for MG6).

Other potential mechanisms. We found that overexpression of *sodB*, an iron-superoxide dismutase, significantly increased *n*-butanol tolerance in *E. coli*. Overexpression of *sodB* has also been found in response to different aromatic substrates and phenol-induced stress in *P. putida* (15, 35, 36), as well as organic solvent stress response in the denitrifying bacteria *Aromatoleum aromaticum* (16, 37). Oxidative stress response has been previously associated with *n*-butanol stress response in *E. coli*, particularly the overexpression of *sodA*, *sodC*, and *yqhD* (6, 10, 17). In fact, the *n*-butanol stress response has been connected with different well-

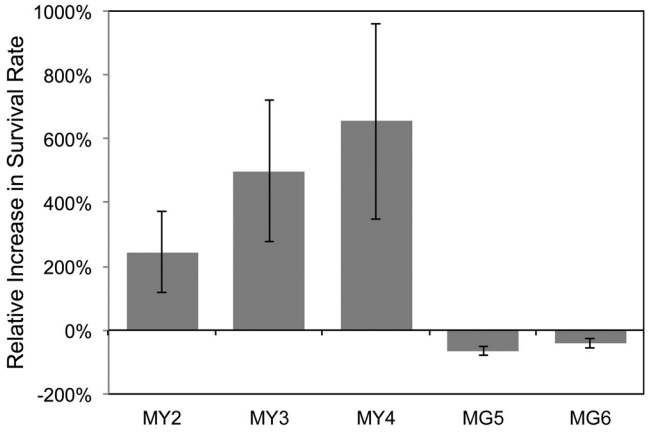

FIG 3 Relative changes in survival rates in osmotic stress. *E. coli* mutants from the YFP-labeled lineage showed increased tolerance to osmotic stress. All the values presented here are statistically significantly different compared with their respective parental strain ($P < 0.05$). Error bars represent the standard deviations.

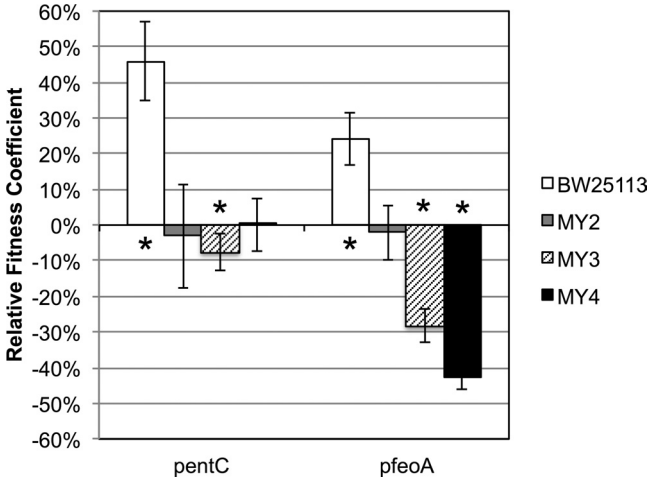

FIG 4 Relative fitness coefficients of the YFP-labeled mutants overexpressing *entC* and *feoA* genes in the presence of 0.8% (vol/vol) *n*-butanol. The fitness coefficients were calculated using the equation $s = (\mu_{strain\_overexpressing\_gene}/\mu_{strain}) - 1$. The asterisks represent statistically significant ($P < 0.05$) values. Error bars represent the standard deviations.

studied stress responses, including oxidative stress response, heat shock, cell envelope stress, and perturbations of different respiratory functions (6, 10, 18).

The proteinase PepB, found to increase *n*-butanol tolerance by more than 40% when overexpressed, has been discovered to be significantly downregulated during acid stress in *Streptococcus* mutants (19, 38). Other mechanisms of protein folding and degradation have been observed in response to *n*-butanol stress, such as activation of the GroESL chaperone system in *E. coli* (11, 19, 39) and *C. acetobutylicum* (12).

Under *n*-butanol exposure, Rutherford et al. (10) observed upregulation of the *nuo* and *cyo* operons, indicating either an increased energy requirement or impairment of respiration. Studies have demonstrated that these genes are necessary for the generation of proton gradients across the inner membrane for aerobic energy generation (40). Here, we showed an increase of more than 23% in fitness relative to that of the wild type under *n*-butanol stress when *nuoI* was overexpressed.

Divergence in evolutionary trajectories for enhanced *n*-butanol tolerance in *E. coli*. The observed differences in the phenotypic profiles between the YFP-labeled and GFP-labeled mutants demonstrated a potential divergence in *n*-butanol tolerance mechanisms between the two lineages. We next asked the question whether the two different mechanisms could be combined to generate a more *n*-butanol-tolerant strain. The iron transport-related genes *feoA* and *entC* were found to be involved in *n*-butanol tolerance in the GFP-labeled mutants (7). These two genes were individually overexpressed in the YFP-labeled mutants, and the levels of *n*-butanol tolerance of the resulting strains were assessed. As shown in Fig. 4, in several cases, the relative fitness coefficient decreased significantly with the expression of the iron-related genes in the YFP-labeled mutants, suggesting an antagonistic relationship between the *n*-butanol tolerance mechanisms in the two differentially colored lineages. Overexpression of *feoA*, involved in the cellular uptake of iron, showed the higher antagonistic effect on the YFP-labeled mutants, where the fitness advantage of MY3 and MY4 in the presence of *n*-butanol decreased significantly (negative fitness coefficients were observed). In the case of *entC*, which is involved in the biosynthesis of enterobactin, a decrease in the relative fitness of MY3 (fitness was unchanged in MY2 and MY4) was observed in the presence of *n*-butanol when overexpressed.

DISCUSSION

In this study, we demonstrated a case of incompatibility between the mechanisms of *n*-butanol tolerance between two different lineages from the same evolved population and identified cross-resistance and antagonistic pleiotropy between different complex phenotypes in the isolated mutants of *E. coli* evolved under *n*-butanol stress. By using VERT, we were able to systematically track the evolutionary dynamics to distinguish parallel mechanisms of *n*-butanol tolerance in the same population, which would otherwise be difficult to determine. Our results indicate that adaptation (increased *n*-butanol tolerance) of *E. coli* to increasing concentrations of *n*-butanol from a single evolving population resulted in two independent and divergent adaptive mechanisms.

The YFP-labeled mutant, MY4, was the only strain that showed a general solvent tolerance to *n*-butanol, isobutanol, and ethanol (see Table 1). Interestingly, MY4 showed a higher fitness advantage in the presence of ethanol than in the presence of *n*-butanol, suggesting the molecular mechanisms for *n*-butanol tolerance in this strain is more general toward solvent stress. Whole-genome sequencing of MY4 led to the identification of a single nucleotide polymorphism in *nusA* (nusAE212A), as we reported previously (7). NusA interacts with several proteins involved in transcriptional termination, including *rho*, a transcriptional terminator affecting expression at the whole-cell level. Point mutation in *rho* has been shown to be a major contributor to ethanol tolerance in *E. coli* evolved under ethanol stress (21). Mutations in global regulators have also been shown to enhance ethanol tolerance and *n*-butanol tolerance (41–43). Thus, the observed mutation in *nusA*, in part, potentially explains the generalist behavior of this mutant toward different stressors by globally perturbing transcriptional regulation in the cell. On the other hand, the GFP-labeled mutants exhibited much higher relative fitness coefficients in the presence of *n*-butanol than in the presence of any other

stress conditions tested, suggesting that GFP-labeled mutants had evolved a more "specialist" adaptive mechanisms for *n*-butanol tolerance. However, MG5 and MG6 (the GFP-labeled mutants) both share the same point mutation in *rho* in addition to an IS5 insertion in *feoA* (presented in Table 3 from our previous work [7]). As *feoA* has been confirmed as a likely genetic determinant for enhanced *n*-butanol tolerance in the GFP-labeled mutants, the results currently suggest that the downstream effects of increased expression of iron uptake-related genes is dominant over any potential downstream effects of the *rho* mutation; further investigation is needed to decipher the respective effects of each mutation in these strains.

The phenotypic response of the alleles under different stress conditions tested indicates divergence in the acquired mechanisms to increase *n*-butanol tolerance between the isolated mutants from the two lineages. Opposite trends in fitness under several conditions were observed. The most notable one is the differential responses to osmotic stress. Under osmotic stress, all the isolated mutants from the yellow lineage showed improved tolerance, while mutants from the green lineage showed an opposite response (Fig. 3). In addition, Reyes et al. (7) documented a cross-tolerance between the cationic antibiotic peptide polymyxin B and *n*-butanol in only the GFP-labeled mutants. This differential cross-resistance in mutants from the two lineages indicates the possibility of diverse mechanisms of *n*-butanol tolerance in *E. coli*. Furthermore, the mechanisms of *n*-butanol tolerance between the two lineages appear to be antagonistic, as the overexpression of *feoA* into MY2 or MY4 resulted in a significant decrease in relative fitness in the presence of *n*-butanol. A similar phenomenon was observed previously between different lineages from a yeast population evolved in glucose-limited conditions (44).

Conclusion. The use of microbial systems in the production of chemicals and fuels from sustainable feedstock requires robust biocatalysts. As the production environment potentially involves multiple inhibitory factors, knowledge regarding the cross-tolerance/antagonistic pleiotropy between different conditions of an adaptive allele becomes important. This work further characterized isolated evolved mutants from a single *n*-butanol-challenged population, identified additional genetic determinants involved in *n*-butanol tolerance in *E. coli*, and discovered several cases of cross-tolerance/antagonistic pleiotropy between different stressors in the isolated mutants and a case of negative epistasis between mechanisms of *n*-butanol tolerance between independent lineages from the same population. Our results suggest that while MG5 and MG6 are our best performers under *n*-butanol stress, these two mutants may not perform as well as MY2 or MY4 in a production environment, thus highlighting the importance of characterizing the effects of identified genetic determinants under multiple relevant conditions to better guide strain engineering efforts.

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REFERENCES

1. **Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC.** 2008. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab. Eng. **10:**305–311.
2. **Berezina OV, Zakharova NV, Brandt A, Yarotsky SV, Schwarz WH, Zverlov VV.** 2010. Reconstructing the clostridial *n*-butanol metabolic pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. **87:**635–646.
3. **Nielsen DR, Leonard E, Yoon S-H, Tseng H-C, Yuan C, Prather KLJ.** 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metab. Eng. **11:**262–273.
4. **Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD.** 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. Microb. Cell Fact. **7:**36.
5. **Dürre P.** 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. **49:**639–648.
6. **Reyes LH, Almario MP, Kao KC.** 2011. Genomic library screens for genes involved in n-butanol tolerance in Escherichia coli. PLoS One **6:**e17678. doi:10.1371/journal.pone.0017678.
7. **Reyes LH, Almario MP, Winkler J, Orozco MM, Kao KC.** 2012. Visualizing evolution in real time to determine the molecular mechanisms of *n*-butanol tolerance in *Escherichia coli*. Metab. Eng. **14:**579–590.
8. **Winkler J, Rehmann M, Kao KC.** 2010. Novel *Escherichia coli* hybrids with enhanced butanol tolerance. Biotechnol. Lett. **32:**915–920.
9. **Minty JJ, Lesnefsky AA, Lin F, Chen Y, Zaroff TA, Veloso AB, Xie B, McConnell CA, Ward RJ, Schwartz DR, Rouillard J-M, Gao Y, Gulari E, Lin XN.** 2011. Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in Escherichia coli. Microb. Cell Fact. **10:**18.
10. **Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD.** 2010. Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl. Environ. Microbiol. **76:**1935–1945.
11. **Zingaro KA, Papoutsakis ET.** 2013. GroESL overexpression imparts *Escherichia coli* tolerance to *i*-, *n*-, and 2-butanol, 1,2,4-butanetriol and ethanol with complex and unpredictable patterns. Metab. Eng. **15:**196–205.
12. **Tomas CA, Welker NE, Papoutsakis ET.** 2003. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl. Environ. Microbiol. **69:**4951–4965.
13. **Borden JR, Papoutsakis ET.** 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **73:**3061–3068.
14. **Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H.** 2006. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. DNA Res. **12:**291–299.
15. **Winkler J, Kao KC.** 2011. Transcriptional analysis of *Lactobacillus brevis* to *n*-butanol and ferulic acid stress responses. PLoS One **6:**e21438. doi:10.1371/journal.pone.0021438.
16. **Nagakubo S, Nishino K, Hirata T, Yamaguchi A.** 2002. The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. J. Bacteriol. **184:**4161–4167.
17. **Kieboom J, de Bont J.** 2001. Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. Microbiology **147:**43–51.
18. **Takatsuka Y, Chen C, Nikaido H.** 2010. Mechanism of recognition of compounds of diverse structures by the multidrug efflux pump AcrB of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **107:**6559–6565.
19. **Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A.** 2011. Engineering microbial biofuel tolerance and export using efflux pumps. Mol. Syst. Biol. **7:**487.
20. **Alsaker KV, Paredes C, Papoutsakis ET.** 2010. Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*. Biotechnol. Bioeng. **105:**1131–1147.
21. **Goodarzi H, Bennett BD, Amini S, Reaves ML, Hottes AK, Rabinowitz JD, Tavazoie S.** 2010. Regulatory and metabolic rewiring during laboratory evolution of ethanol tolerance in *E. coli*. Mol. Syst. Biol. **6:**378.
22. **Purvis JE, Yomano LP, Ingram LO.** 2005. Enhanced trehalose produc-

tion improves growth of *Escherichia coli* under osmotic stress. Appl. Environ. Microbiol. **71:**3761–3769.
23. **Sun Y, Vanderpool CK.** 2011. Regulation and function of *Escherichia coli* sugar efflux transporter A (SetA) during glucose-phosphate stress. J. Bacteriol. **193:**143–153.
24. **Liu JY, Miller PF, Willard J, Olson ER.** 1999. Functional and biochemical characterization of *Escherichia coli* sugar efflux transporters. J. Biol. Chem. **274:**22977–22984.
25. **Zhang M, Mileykovskaya E, Dowhan W.** 2002. Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J. Biol. Chem. **277:**43553–43556.
26. **Mileykovskaya E, Dowhan W.** 2009. Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim. Biophys. Acta **1788:**2084–2091.
27. **Romantsov T, Stalker L, Culham DE, Wood JM.** 2008. Cardiolipin controls the osmotic stress response and the subcellular location of transporter ProP in *Escherichia coli*. J. Biol. Chem. **283:**12314–12323.
28. **Haines TH.** 2009. A new look at cardiolipin. Biochim. Biophys. Acta **1788:**1997–2002.
29. **Erez E, Stjepanovic G, Zelazny AM, Brugger B, Sinning I, Bibi E.** 2010. Genetic evidence for functional interaction of the *Escherichia coli* signal recognition particle receptor with acidic lipids *in vivo*. J. Biol. Chem. **285:**40508–40514.
30. **Ma R, Zhang Y, Hong H, Lu W, Lin M, Chen M, Zhang W.** 2011. Improved osmotic tolerance and ethanol production of ethanologenic *Escherichia coli* by IrrE, a global regulator of radiation-resistance of *Deinococcus radiodurans*. Curr. Microbiol. **62:**659–664.
31. **Balakumar S, Arasaratnam V.** 2012. Osmo-, thermo- and ethanol- tolerances of *Saccharomyces cerevisiae* S1. Braz. J. Microbiol. **43:**157–166.
32. **Okolo BN, Moneke AN, Anyanwu CU, Ezeogu LI, Aligwekwe GN.** 2004. On the pH and osmotic stress tolerance of high ethanol tolerant palm wine *Saccharomyces* yeast isolates. Bio-Research **2:**1–7.
33. **Dragosits M, Mozhayskiy V, Quinones-Soto S, Park J, Tagkopoulos I.** 2013. Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*. Mol. Syst. Biol. **9:**643.
34. **Nicolaou SA, Gaida SM, Papoutsakis ET.** 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metab. Eng. **12:**307–331.
35. **Kim YH, Cho K, Yun S-H, Kim JY, Kwon K-H, Yoo JS, Kim SI.** 2006. Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomic approach: 2-DE/MS and cleavable isotope-coded affinity tag analysis. Proteomics **6:**1301–1318.
36. **Santos PM, Benndorf D, Sá-Correia I.** 2004. Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. Proteomics **4:**2640–2652.
37. **Trautwein K, Kühner S, Wöhlbrand L, Halder T, Kuchta K, Steinbüchel A, Rabus R.** 2008. Solvent stress response of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1. Appl. Environ. Microbiol. **74:**2267–2274.
38. **Len ACL, Harty DWS, Jacques NA.** 2004. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. Microbiology **150:**1339–1351.
39. **Zingaro KA, Papoutsakis ET.** 2012. Toward a semisynthetic stress response system to engineer microbial solvent tolerance. mBio **3**(5): e00308–12. doi:10.1128/mBio.00308-12.
40. **Lennen RM, Kruziki MA, Kumar K, Zinkel RA, Burnum KE, Lipton MS, Hoover SW, Ranatunga DR, Wittkopp TM, Marner WD, Pfleger BF.** 2011. Membrane stresses induced by overproduction of free fatty acids in *Escherichia coli*. Appl. Environ. Microbiol. **77:**8114–8128.
41. **Klein-Marcuschamer D, Santos CNS, Yu H, Stephanopoulos G.** 2009. Mutagenesis of the bacterial RNA polymerase alpha subunit for improvement of complex phenotypes. Appl. Environ. Microbiol. **75:**2705–2711.
42. **Alper H, Stephanopoulos G.** 2007. Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab. Eng. **9:**258–267.
43. **Santos CNS, Stephanopoulos G.** 2008. Combinatorial engineering of microbes for optimizing cellular phenotype. Curr. Opin. Chem. Biol. **12:**168–176.
44. **Kvitek DJ, Sherlock G.** 2011. Reciprocal sign epistasis between frequently experimentally evolved adaptive mutations causes a rugged fitness landscape. PLoS Genet. **7:**e1002056. doi:10.1371/journal.pgen.1002056.