

Enhancing Tolerance to Short-Chain Alcohols by Engineering the *Escherichia coli* AcrB Efflux Pump to Secrete the Non-native Substrate *n*-Butanol

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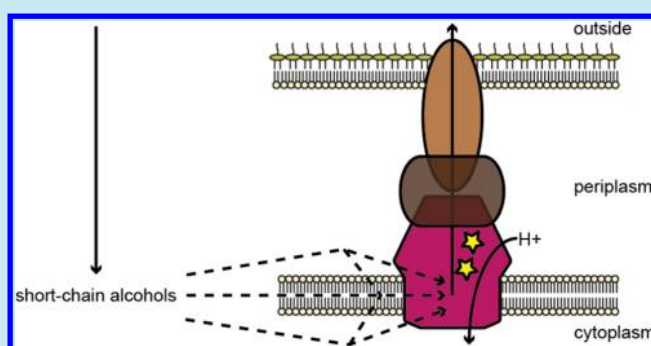
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Supporting Information

ABSTRACT: The microbial conversion of sugars to fuels is a promising technology, but the byproducts of biomass pretreatment processes and the fuels themselves are often toxic at industrially relevant levels. One promising solution to these problems is to engineer efflux pumps to secrete fuels and inhibitory chemicals from the cell, increasing microbial tolerance and enabling higher fuel titer. Toward that end, we used a directed evolution strategy to generate variants of the *Escherichia coli* AcrB efflux pump that act on the non-native substrate *n*-butanol, enhancing growth rates of *E. coli* in the presence of this biofuel by up to 25%. Furthermore, these variants confer improved tolerance to isobutanol and straight-chain alcohols up to *n*-heptanol. Single amino acid changes in AcrB responsible for this phenotype were identified. We have also shown that both the chemical and genetic inactivation of pump activity eliminate the tolerance conferred by AcrB pump variants, supporting our assertion that the variants secrete the non-native substrates. This strategy can be applied to create an array of efflux pumps that modulate the intracellular concentrations of small molecules of interest to microbial fuel and chemical production.

KEYWORDS: directed evolution, protein engineering, biofuels, tolerance, efflux pump, transporter



The development of advanced cellulosic biofuels promises to positively impact global climate change, rural development, and energy security.^{1–3} Engineered microbes are being used to convert plant biomass into fuels and chemicals.^{4–7} However, the toxicity of some of the fuel products to the microbial production host remains a key scientific challenge to the process.^{8–11} Biofuel toxicity limits the growth of bacterial and yeast production strains, which in turn limits biofuel titers.^{8,12} A number of strategies have been pursued to reduce the toxicity of biofuels to cells, including overexpression of heat shock proteins, modification of the cell membrane, and alteration of the cellular stress response.^{13,14} However, it is perhaps most desirable to actively efflux biofuels using membrane transporters, thus reducing toxic effects and potentially improving biofuel recovery.^{15–18} Additionally, many fuel-producing reactions in the cell are reversible and may therefore proceed in either the forward or reverse direction.¹⁹ By pumping the product out of the intracellular

milieu, such reversible biofuel production reactions will be pulled in the forward direction, providing an additional increase in productivity. Unfortunately, most biofuel candidates are either not known substrates for microbial transporters²⁰ or are not secreted at relevant rates.^{21,22}

The RND (resistance-nodulation-division) family of efflux pumps is an attractive starting point for the development of pumps that act on biofuel-like molecules. RND-type efflux pumps are native to Gram-negative bacteria, including *Escherichia coli*, and have a wide range of substrates.^{23,24} Moreover, RND-type pumps are powered by the proton gradient rather than ATP and have the potential to secrete compounds directly from the cytoplasm, inner membrane, and periplasm to the extracellular space.²⁵ As depicted in Figure 1a,

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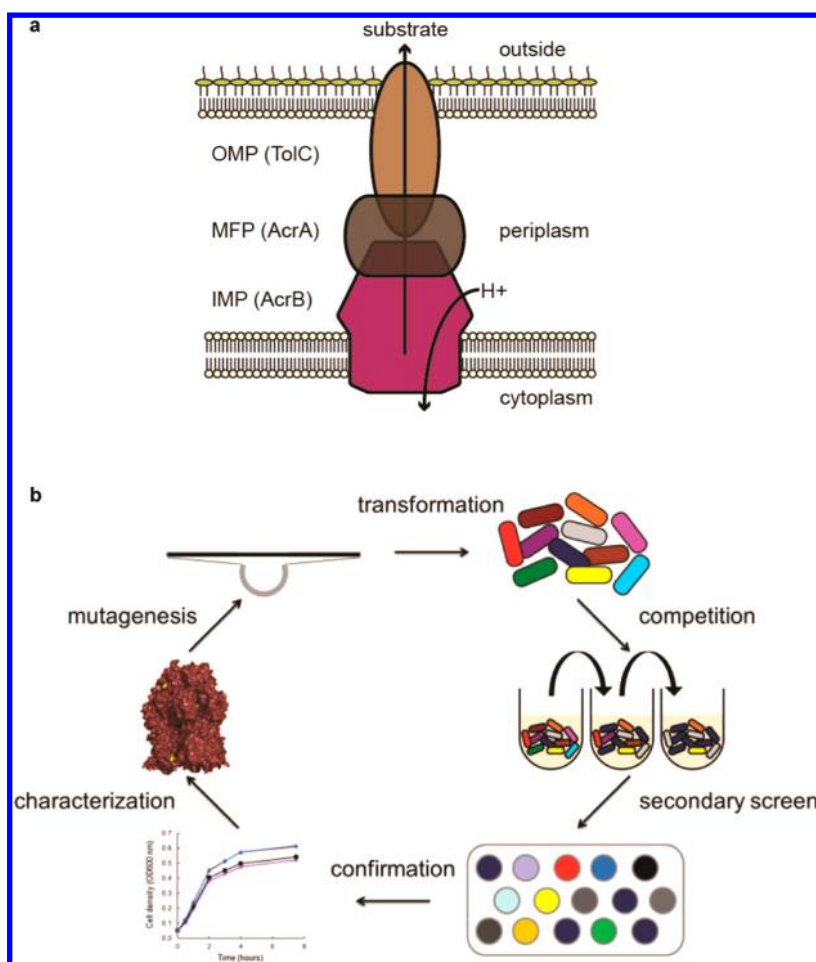


Figure 1. Directed evolution approach used to generate tolerance-conferring variants of the AcrB efflux pump. (a) Schematic of the AcrAB-TolC tripartite pump system. The *E. coli* AcrB protein is an inner membrane efflux pump that works in concert with a membrane fusion protein (AcrA) and outer membrane protein (TolC). AcrB has been implicated in substrate recognition and is a proton antiporter. (b) *E. coli* transformed with libraries of AcrB variants were subjected to exponential growth competition in the presence or absence of 0.5% *n*-butanol. After the competition, cells from both pools were plated, and individual clones were tested for growth performance in *n*-butanol. After confirming AcrB variant-conferred growth, another round of mutation and selection was performed.

these pumps are composed of an outer membrane channel (OMP), membrane fusion protein (MFP), and inner membrane pump (IMP).²⁶ The inner membrane pump has been implicated as the major unit responsible for substrate recognition and extrusion of the compounds.^{27,28}

Two recent studies have explored the heterologous expression of transporters in a model organism for bacterial biofuel production, *E. coli*.^{20,29} Dunlop et al. screened a panel of 43 RND-type efflux pumps for those that enhanced tolerance to exogenously added biofuels.²⁰ They identified transporters that enhanced tolerance to geranyl acetate, geraniol, α -pinene, limonene, and farnesyl hexanoate and demonstrated that the isolated efflux pump conferred ~ 1.5 -fold limonene secretion increase in a producing strain. However, none of the 43 transporters enhanced tolerance to *n*-butanol or isopentanol, highly attractive biofuels. Building on this approach, Doshi, Nguyen, and Chang screened 16 ABC (ATP-binding cassette) transporters for those that enhanced secretion of zeaxanthin, canthaxanthin, β -carotene, or lycopene from isoprenoid-producing strains.²⁹ Secretion of these molecules was enhanced ~ 2.5 - to 4.5-fold, again demonstrating the validity of the strategy.

Furthermore, directed evolution has been applied to manipulate the functionality of transporter systems. When an operon conferring resistance to arsenic was evolved by DNA shuffling, it was found that mutations in an inner membrane permease component contributed a 4- to 6-fold increase in arsenite tolerance to *E. coli*.³⁰ The RND transporter genes have been evolved to increase the tolerance of *E. coli* to antibiotics.^{31,32} Very recently, the *E. coli* IMP AcrB was evolved by Foo and Leong to achieve increased secretion of its native substrate *n*-octane.²² Interestingly, the isolated variants of AcrB in this report also conferred enhanced tolerance to another native AcrB substrate, α -pinene, indicating that the evolution gave rise to a more active pump, but not one with increased specificity to novel substrates.

We show that RND-type pumps can be engineered to act on non-natural substrates and confer greater tolerance to many short-chain alcohols. AcrB from *E. coli* is one of the most well-characterized inner membrane efflux pump proteins from the RND family.²⁴ In this study, we have created libraries of the wild-type AcrB (wtAcrB) efflux pump transporter, expressed these libraries in *E. coli*, and selected AcrB variants that enhance tolerance when cells are grown in the presence of *n*-butanol. Two rounds of directed evolution resulted in AcrB variants that

increase log-phase growth by up to 25% in the presence of *n*-butanol. We have also shown that these AcrB variants enhance tolerance to a number of short-chain alcohols, but not *n*-octanol, 3-octanol or native AcrB substrates such as ampicillin and chloramphenicol. The AcrB variants do not confer increased growth rates or cell density in media in the absence of these toxic solvents. Individual amino acid changes were found to be responsible for tolerance enhancement conferred by the majority of variants. Importantly, chemical and genetic methods indicate that our AcrB variants actively transport alcohols out of the cell. To our knowledge, this work is the first example in which an RND transporter system has been evolved in the laboratory for the secretion of non-native toxic compounds.

RESULTS AND DISCUSSION

Generation of Initial Library of Mutagenized *acrB*. We chose to use a directed evolution strategy (Figure 1b) to alter the substrate specificity of the wtAcrB efflux pump from *E. coli*. The entire *acrB* gene was subjected to random mutagenesis and incorporated into a vector under the control of the *araBAD* promoter³³ along with the *acrA* and *tolC* genes, which encode the other two proteins of the tripartite pump. The library comprises 50 000 variants with an average error rate of 0.185%. The three genes of the plasmid-encoded operon (pBAD*tolC*-*acrAB*) were observed to be expressed at the same order of magnitude (data not shown).

Selection via Growth Competition in *n*-Butanol Resulted in Enhanced Tolerance to Alcohols. The *acrB* library was subjected to a selection to identify those library members that conferred enhanced growth in the presence of *n*-butanol. Specifically, *E. coli* cells harboring the library of variants were subjected to an exponential growth competition in the absence (control) or presence (challenged) of 0.5% *n*-butanol. After three rounds of log phase growth followed by serial dilution, dilutions of the remaining cellular populations were plated, and 30 clones from the challenged population and 12 clones from the control population were chosen for further characterization. After back transformation of naïve *E. coli* DH10B with the plasmids, we carried out growth assays under optimized conditions at 0.7% *n*-butanol. Two positive variants were identified from the challenged population: Var1 (Ile370Thr, Ile466Phe, Gly689AspD, Ala942Thr) and Var2 (Glu130Gly, Glu269Gly, Met355Leu, Val448Ala, Thr495Ala) (Figure 2). In the presence of *n*-butanol, these variants conferred growth to higher cell densities (30% and 25% more after six hours of growth for Var1 and Var2, respectively) as compared to cells expressing wtAcrB. All strains grew the same in the absence of inhibitor (Figure S1a, Supporting Information). Interestingly, two libraries targeting residues in the periplasmic loop regions of wtAcrB, which have been implicated as substrate-binding determinants,^{27,34} did not give rise to any improved variants using the same selection strategy.

We next examined the effect of the two positive variants on growth of *E. coli* in the presence of other toxic small molecules. Compared to wtAcrB and empty vector, Var1 and Var2 also conferred varying levels of enhanced growth, as measured by higher cell densities (and, in some cases, increased log-phase growth rates) to cells grown in the presence of 6% ethanol, 0.8% isobutanol, 0.2% *n*-pentanol, 0.1% *n*-hexanol, and 0.04% *n*-heptanol, but not 0.013% *n*-octanol, 0.035% 3-octanol, 2.1 mg/L chloramphenicol, or 20 mg/L ampicillin (Figure 3; empty vector and 3-octanol data not shown). The variants also

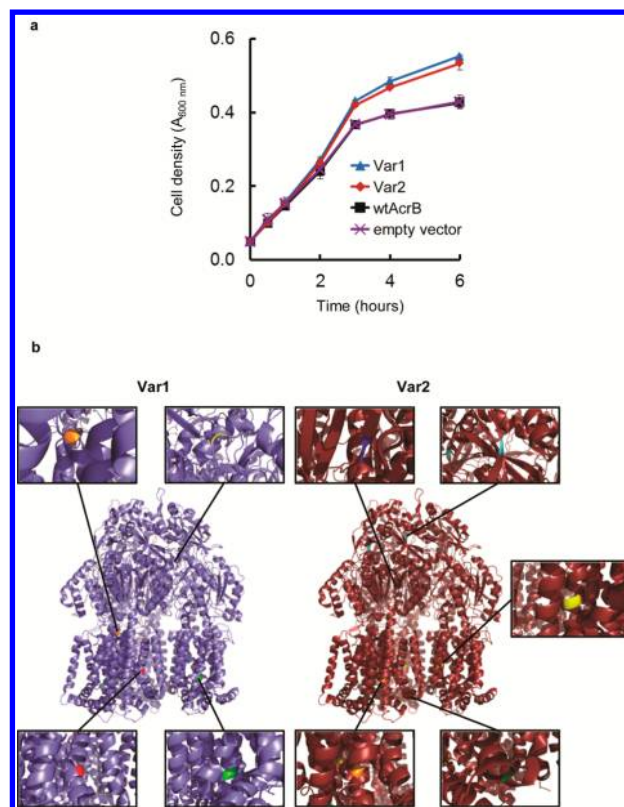


Figure 2. AcrB variants Var1 and Var2 confer enhanced growth in the presence of *n*-butanol. (a) Plot of cell growth with respect to time for strains harboring empty vector and wtAcrB controls, Var1, and Var2, in the presence of 0.7% *n*-butanol. Error bars represent one standard deviation of three independent experiments. (b) Models of AcrB variants. The locations of the mutated residues are noted on a published structure of AcrB. Var1: Ile370Thr (red), Ile466Phe (orange), Gly689AspD (yellow), Ala942Thr (green). Var2: Glu130Gly (blue), Glu269Gly (cyan), Met355Leu (orange), Val448Ala (yellow), Thr495Ala (green). PDB entry 2DRD was manipulated using PyMOL.

did not confer increased tolerance to *n*-octanol at concentrations of 0.005%, 0.012%, or 0.023% after 24 h (data not shown). Increasing the concentration of inducer reduced the growth of cells harboring both variants and wtAcrB and did not result in a greater difference in growth between cells expressing Var1, Var2, and the controls in the presence of 0.7% *n*-butanol (Figure S1b, Supporting Information). Furthermore, AcrB variants maintain tolerance enhancement over the range of *n*-butanol concentrations tested (from 0.33 to 0.8%, data not shown).

A Second Round of Mutagenesis and Selection Further Increased Tolerance to *n*-Butanol and Isobutanol. Using the gene encoding Var1 as the parent gene for a second round of whole-gene mutagenesis, we generated a library of 65 000 variants with an average error rate of 0.09%. This second-generation library was subjected to an exponential growth competition in media supplemented with *n*-butanol as well as media supplemented with isobutanol, and a total of 33 clones were tested in the secondary screens. No additional variants with improved tolerance were obtained from the second-round selection in *n*-butanol. From the isobutanol selection, two more variants, Var1-1 and Var1-2, were identified with improved tolerance over Var1 (Figure 4). Notably, compared to wtAcrB, Var1-2 confers a 25% increase in the

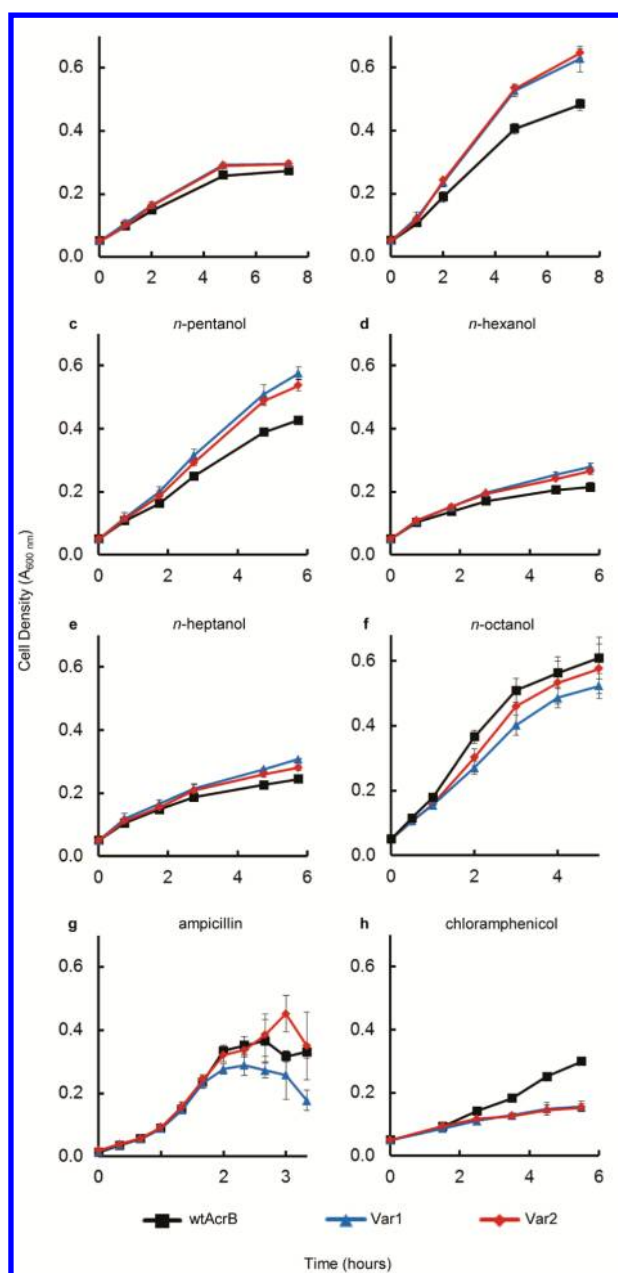


Figure 3. Var1 and Var2 confer enhanced growth in the presence of many short-chain alcohols, but not *n*-octanol or the antibiotics ampicillin and chloramphenicol. Plots of cell growth with respect to time in medium supplemented with different inhibitors. In 6% ethanol (a), 0.8% isobutanol (b), 0.2% *n*-pentanol (c), 0.1% *n*-hexanol (d), and 0.04% *n*-heptanol (e), Var1- and Var2-expressing strains grow to a greater final cell density than cells expressing AcrB, or harboring empty vector (data not shown). In growth medium supplemented with 0.013% *n*-octanol (f), 20 mg/L ampicillin (g), or 2.1 mg/L chloramphenicol (h), there is no growth advantage conferred by Var1 or Var2. Error bars represent one standard deviation. Cells exposed to ampicillin were grown in triplicate in microtiter plates; all other experiments were carried out in triplicate in screw-cap test tubes.

late log-phase first-order growth rate constant and a 37% higher cell density after six hours for cells grown in *n*-butanol. We also performed inhibition experiments as described by Dunlop et al. 2011.²⁰ As shown in Figure S2, Supporting Information, cells harboring AcrB pump variants Var1 and Var1-2 maintain higher cell culture densities after 14 h of growth in 0.33, 0.45, and

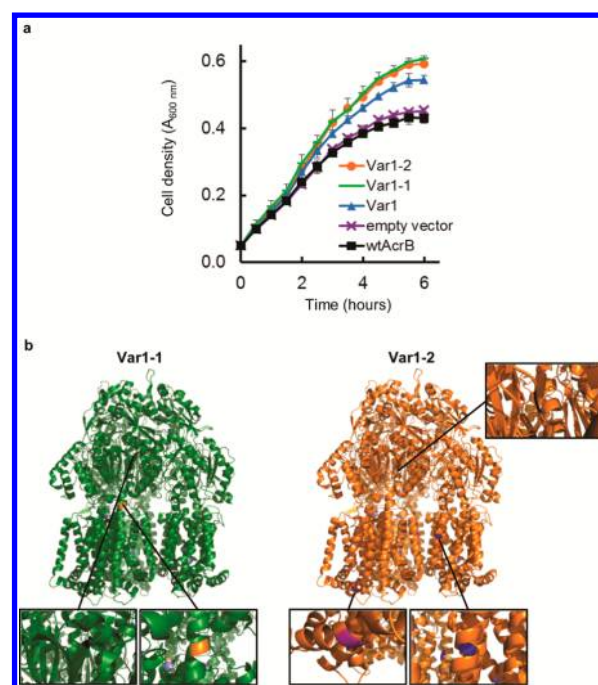


Figure 4. Second-generation AcrB efflux pump variants Var1-1 and Var1-2 further enhance tolerance to *n*-butanol. (a) Growth curves for strains harboring empty vector, wtAcrB, Var1, Var1-1, and Var1-2, grown in medium supplemented with 0.7% *n*-butanol. Error bars represent one standard deviation of three independent experiments. (b) Models of AcrB variants. The additional Var1-1 mutations include Asn144Ser (black), Ile335Thr (orange), and Ser1043Arg (residue 1043 does not appear in the published structure 2DRD); additional Var1-2 mutations include Asp174Asn (black), Lys522Gly (purple), and Ser880Pro (blue). Var1 mutations are colored light blue. PDB entry 2DRD was manipulated using PyMOL.

0.55% *n*-butanol (v/v) than cells harboring the AcrB or empty vector controls. The additional Var1-1 mutations include Asn144Ser, Ile335Thr, and Ser1043Arg; additional Var1-2 mutations include Asp174Asn, Lys522Gly, and Ser880Pro. It should be noted that none of the mutations significantly altered expression of the variants compared to expression of wild-type AcrB (Figure S3, Supporting Information).

Butanol Concentration within Cells Is Lower for Cells Expressing Var1-2 than for Cells Expressing Wild Type AcrB

To verify that the growth improvements are due to an increased efflux of *n*-butanol, we set out to determine whether the intracellular level of butanol is altered by the presence of the variants as compared to wtAcrB. To do so, we incubated cells containing either wtAcrB or Var1-2 with 0.6% *n*-butanol and let them equilibrate for 1 h. We then measured the amount of *n*-butanol remaining in the buffer and calculated the amount retained by the cells. If the growth phenotype is due to increased *n*-butanol efflux, we would expect to observe higher concentrations of *n*-butanol in the medium incubated with the cells expressing the evolved Var1-2 as compared to wtAcrB, translating into a lower concentration within cells harboring Var1-2. We indeed detected an approximately 15% decrease in *n*-butanol sequestration by the cells expressing Var1-2 over cells expressing wtAcrB ($0.552 \pm 0.046 \mu\text{g/mL}$ for Var1-2 versus $0.652 \pm 0.020 \mu\text{g/mL}$ for wtAcrB, $p < 0.01$ by two-tailed student's *t* test).

It is as-yet unknown how much improvement in tolerance is required for commercial feasibility, as we do not know what

amount of *n*-butanol is toxic within the cytoplasm of *E. coli*, nor what concentration will be present in cells producing the *n*-butanol. However, our results indicate that intracellular concentration of *n*-butanol is indeed lower in the presence of our top-performing AcrB variant, a result that we expect would be mirrored in butanol-production strains and thus would impact resulting titer.

Individual Mutations Are Responsible for Growth Enhancement in the Presence of *n*-Butanol. Multiple mutations were observed in all of the variants that enhance growth in the presence of alcohols. To determine which mutations are required for tolerance enhancement, we created each individual mutation found in Var1 and Var2, resulting in nine variants of AcrB mutated at single residues. These subvariants were then subjected to the *n*-butanol growth assay. It was found that Ile466Phe is wholly responsible for Var1-enhanced growth in *n*-butanol (Figure 5a), and Met355Leu is responsible for Var2-enhanced growth (Figure 5b). In analogous experiments with the second generation variants, we created each individual mutation from Var1–1 and Var1–2 in the Var1 background. The Var1–2 Ser880Pro mutation was found to be the major contributor to variant-enhanced growth in *n*-butanol (Figure 5c), while no single Var1–1 mutation was identified to be solely responsible for the tolerance enhancement.

The mechanisms by which mutations in AcrB and homologous pump proteins are proposed to affect transport are difficult to reconcile without experimentally determined crystal structures.^{22,32,35} AcrB is a homotrimer in the assembled pump structure, and rotates through conformations termed “access”, “binding”, and “extrusion” as it effluxes a substrate.^{36,37} All three of the contributing mutations identified in our study (Ile466Phe, Met355Leu, and Ser880Pro) are found in transmembrane helices of AcrB and are in regions of the protein that undergo dramatic conformational changes in the access protomer of the homotrimer.^{36,37} Ile466 is at the periplasmic side of transmembrane helix 5 and lines the AcrB central cavity (Figure S4a, Supporting Information). In the binding and extrusion protomers of PDB entry 2DRD, Ile466 is positioned in the α -helix 1.5 from the helix terminus. However, in the access protomer of 2DRD, Ile466 is modeled as the terminal residue of transmembrane helix 5. The mutation of Ile466 to Phe, a larger and more nonpolar residue, is likely to cause an even greater conformational change in the Ile466Phe variant to adjust for steric clashes and water organization if this residue is exposed to the periplasmic environment. As it lines the central cavity, it may also interact with alcohols, contributing to the transport of the compound through AcrB.

Met355 is located on the cytoplasmic side of transmembrane helix 2 (Figure S4b, Supporting Information). In the binding and extrusion protomers, it is half a turn from the terminus. In the access protomer of 2DRD, it is immediately at the terminus. This mutation may disrupt packing and global helix arrangement in the membrane, perhaps even creating enough shift to permit substrate entrance to the central cavity from the cytoplasm. However, when wtAcrB was mutated to contain both Ile466Phe and Met355Leu, the double mutant did not give rise to observable additive or synergistic *n*-butanol tolerance. Thus, we conclude that these mutations both result in a broad disruption to multiple transmembrane helices such that it would have been difficult to predict the efflux of butanol using a rational approach.

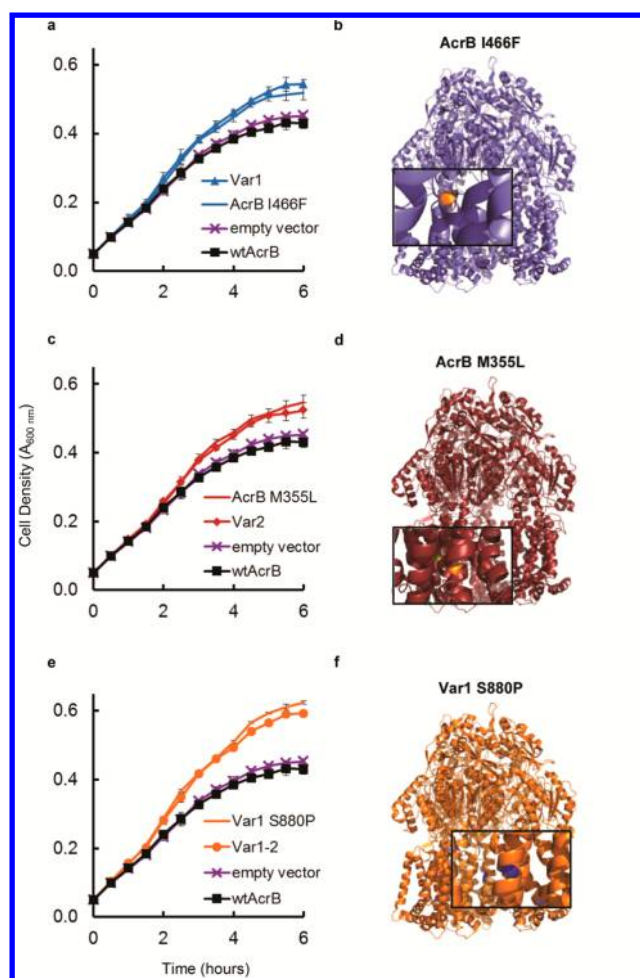


Figure 5. Individual mutations in Var1 (Ile466Phe), Var2 (Met355Leu), and Var1-2 (Ser880Pro) are responsible for growth enhancement in *n*-butanol. (a) Growth curves for strains harboring empty vector, wtAcrB, Var1, and AcrB Ile466Phe, grown in medium supplemented with 0.7% *n*-butanol. (b) Model of AcrB with labeled Ile466Phe (orange). (c) Growth curves for strains harboring empty vector, wtAcrB, Var2, and AcrB Met355Leu, grown in medium supplemented with 0.7% *n*-butanol. (d) Model of AcrB with labeled Met355Leu (orange). (e) Growth curves for strains harboring empty vector, wtAcrB, Var1-2, and Var1 Ser880Pro, grown in medium supplemented with 0.7% *n*-butanol. (f) Model of Var1 with labeled Ser880Pro (blue), based on AcrB structure. Var1 mutations are colored light blue. PDB entry 2DRD was manipulated using PyMOL. In a, c, and e, error bars represent one standard deviation of three independent experiments.

Ser880 is located within transmembrane helix 8 (Figure S4c, Supporting Information). Transmembrane helix 8 is an interrupted α -helix in the binding protomer, a very long α -helix in the extrusion protomer, and a greatly interrupted α -helix in the access protomer. In the access protomer, Ser880 is only one turn from the periplasmic side helix terminus, compared to two turns away in the binding protomer and five turns away in the extrusion protomer. Proline is a known helix breaker, and so the mutation of Ser880 to Pro likely malforms this helix and perhaps disrupts other transmembrane helices as well, akin to the proposed disruptions brought on by the Ile466Phe and Met355Leu mutations.

Elimination of the Proton Motive Force Disrupts the AcrB Variant-Conferred Tolerance Phenotype. The energy that drives the conformational changes and substrate

transport of RND-type efflux pumps is derived from the binding and subsequent release of protons. It is possible to eliminate the proton gradient that exists across the periplasmic membrane, and therefore eliminate the proton motive force, by growing cells in the presence of a proton ionophore.³⁸ Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) binds protons and carries them through lipid bilayers such as the *E. coli* periplasmic membrane.³⁹ When our series of strains was grown in the combined presence of *n*-butanol or isobutanol and CCCP, the AcrB variants no longer conferred a growth advantage to the cell (*n*-butanol data shown in Figure S5, Supporting Information). In fact, the AcrB variants conferred an extended lag phase, which was observed even in growth medium without *n*-butanol or isobutanol. The extended lag phase was followed by log phase growth on par with that of the empty vector and wtAcrB strains, and all strains grew to equal cell densities in stationary phase. This evidence suggests that the activity of the AcrB variants is dependent upon the presence of the proton gradient, and thus that the variants actively transport alcohols out of the cell.

Elimination of the AcrB Proton Relay Functionality Disrupts the AcrB variant-Conferred Tolerance Phenotype. Nikaido and colleagues have identified the wtAcrB residues that are at the wtAcrB proton translocation site; mutating aspartates at amino acid sequence positions 407 and 408 to alanine greatly reduces AcrB activity.^{40,41} We made Asp407Ala Asp408Ala double mutant versions of the wild type AcrB and the tolerance-conferring variants. We hypothesized that in the presence of alcohols, the proton relay-deficient variants would no longer confer tolerance. As shown in Figure 6, we observe growth that is equivalent to that of cells containing the empty vector or expressing wtAcrB for the various D407A D408A strains. As previously observed, the variants with the intact proton relay residues give rise to a higher cell density and, for second-generation variants, increased late log-phase growth rates. This indicates that the binding and subsequent release of a proton, and therefore

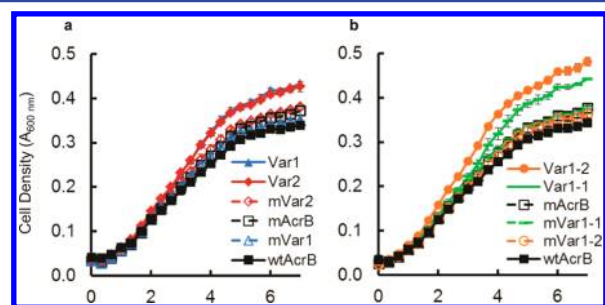


Figure 6. Variant-conferred tolerance is eliminated when the AcrB proton translocation site is mutated. (a) Plot of cell growth with respect to time for generation one AcrB variants. Strains harboring empty vector, wtAcrB, Var1, Var2, mVar1, mVar2, and mVar2 were grown in medium supplemented with 0.4% *n*-butanol in microtiter plates. The empty vector trace was omitted for the sake of clarity, and was very similar to wtAcrB. (b) Plot of cell growth with respect to time for generation two AcrB variants. Strains harboring empty vector, wtAcrB, Var1-1, Var1-2, mAcrB, mVar1-1, and mVar1-2 were grown in medium supplemented with 0.4% *n*-butanol in microtiter plates. The empty vector trace was omitted for the sake of clarity, and was very similar to wtAcrB. The “m” denotes Asp407Ala Asp408Ala double mutant versions of AcrB and the tolerance-conferring variants. In a and b, error bars represent one standard deviation of three independent experiments.

conformational change and active transport, are required to confer tolerance in these strains. Taken together, the experiments with CCCP and the proton-relay mutants support our assertion that the AcrB variants confer enhanced tolerance to *n*-butanol and isobutanol by actively exporting alcohols from the cell.

Low-Level Overexpression of wtAcrB and AcrB Variants Does Not Affect Membrane Integrity.

Membrane protein expression may affect the integrity of the cell. To rule out any such effects on tolerance, experiments evaluating membrane integrity⁴² were carried out. Cells were harvested after log-phase growth, and culture supernatants, 10-fold concentrated culture supernatants, and whole cell lysates were interrogated for the presence of the periplasmic maltose binding protein (MBP) and cytoplasmic chaperone Hsp60 (GroEL). Compared to cells harboring empty vector, none of the cells producing TolC, AcrA, and wtAcrB or AcrB variants exhibit differences in the quantity of MBP and GroEL detected in the supernatants and whole cell lysates (Figure S6a,b, Supporting Information). To assess the presence of any smaller membrane perturbations, we also performed staining with the small molecule propidium iodide, which is not a known AcrB substrate. As quantified by flow cytometry, we observe no difference in propidium iodide uptake among cells containing wtAcrB, the variants, or empty vector, while cells damaged by heat shock demonstrate a considerable increase in fluorescence (Figure S6c, Supporting Information). This shows that the *E. coli* inner and outer membranes are structurally sound with low-level AcrAB-TolC expression, and provides further evidence that the tolerance phenotype we observe is due to the activity of AcrB variants.

AcrB Variants Do Not Confer Enhanced Tolerance in Δ acrB Strains.

For both *E. coli* MG1655 and BW25113, Δ acrB knockout strains grow at a higher rate and to higher final cell densities than the wild-type strains in the presence of the short-chain alcohols *n*-butanol and isobutanol (Figure S7a, Supporting Information). Without these alcohols in the medium, the strains grow at nearly the same rate. Moreover, a recent study using *E. coli* DH1 Δ acrAB that screened a panel of 43 naturally occurring efflux pumps, including AcrAB, did not identify any pumps that enhanced *E. coli* tolerance in the presence of the short-chain alcohols *n*-butanol or isopentanol.²⁰ We therefore suspected that the enhanced growth rate of the Δ acrB strains versus the *acrB*⁺ strains in the presence of inhibitory short-chain alcohols would occlude potential growth enhancements conferred by efflux pumps. This was partially confirmed in our experiments in which we expressed our AcrB efflux pump variants in *E. coli* MG1655 Δ acrB and observed an enhancement of tolerance compared to wtAcrB expressed from the same vector but not compared to the empty vector control. As expected, we observed growth enhancement for our variants compared to all controls in the *E. coli* MG1655 wild-type background (Figure S7b, Supporting Information), with similar phenotypes as in the *E. coli* DH10B strain in which all our other work was conducted.

As described above, our libraries were constructed in *E. coli* strains that retained the wild type *acrB* gene on the chromosome. Since a functional RND efflux pump requires a trimer of AcrB, the presence of wtAcrB permitted the formation of wtAcrB or AcrB variant homotrimers as well as wtAcrB/AcrB variant heterotrimers. However, recent experimental evidence demonstrated that coexpressing AcrB variants is biased in favor of pure trimers versus mixed trimers.⁴³ In addition, our

expression vectors contain a low to medium copy-number origin (from p15A), and so, the resulting expressed protein may be present at significantly higher levels than any background wtAcrB arising from the genome. Thus, we suggest that the majority of efflux pumps are likely to be pure homotrimers, be they wtAcrB or AcrB variant, in our system. Moreover, we are certain that the homotrimers are capable of conferring the enhanced growth phenotype since the variants outperform the wtAcrB in the Δ acrB strain, and only homotrimers can form in this strain.

Summary and Concluding Remarks. With the goals of reducing toxicity, enhancing recovery, and increasing production of microbially derived chemicals, we have used directed evolution to identify variants of the *E. coli* inner membrane pump protein AcrB that enhance the growth of *E. coli* in the presence of alcohols by up to 25% and decrease the amount of exogenously added *n*-butanol taken up by the cells by 15%. Previously, researchers have reported a number of other methods for increasing *n*-butanol tolerance in *E. coli*^{44–48} to various degrees of success. For instance, Zingaro and Papoutsakis⁴⁶ observed as much as a 4-fold increase in cell viability when heat shock proteins were overexpressed, while Kao and co-workers⁴⁷ used overexpression and deletion libraries to identify strain variants that gave rise to approximately 50% enhancement in cell growth. While our variants fall on the lower side in terms of growth rate enhancement, we have shown a fundamentally different mechanism can be successful: mutations in specific proteins that promote secretion of butanol from within the cell rather than prevent its entry or enhance a cell-wide stress response. As such, this method of tolerance lends itself to easy integration with other methods for engineering *E. coli* to be butanol tolerant in a bioprocess setting.

In this study, as well as many others,^{44–48} tolerance has been measured to exogenously added *n*-butanol, but the desired phenotype is for enhanced tolerance to cytosolically produced *n*-butanol. Therefore, it would be of interest to test these alcohol-secreting pumps in the context of *n*-butanol- and other alcohol-producing strains. The results of such studies will provide further insight into the effectiveness of growth selections using exogenously added compounds at toxic concentrations compared to that of screens in production strains.^{12,49} Our study provides proof-of-principle that the engineering of transporters for improved tolerance to exogenous inhibitors is effective and is directly applicable to toxic components of biomass hydrolysates, toxic compounds secreted by another microbe in a coculture, toxic products of metabolic pathways, or compounds that are toxic to a cell in any other constrained environmental context. Importantly, we have demonstrated that directed evolution of membrane transporters can give rise to specificity for non-native substrates, which will undoubtedly be of use to synthetic biologists as a tool for controlling small molecule concentration gradients across the cell.

METHODS

Strains and Bacterial Growth Conditions. *E. coli* strains used in this study are listed in Table S1, Supporting Information. *E. coli* Δ acrB strains were constructed by P1 transduction and antibiotic resistance cassettes removed from the chromosome according to established procedures.⁵⁰ For bacterial growth experiments, Luria–Bertani medium/lysogenic broth (LB) was supplemented with 50 μ g/mL kanamycin

unless otherwise noted. Strains were incubated at 37 °C and, when grown in liquid medium, shaken at 225 rpm. When noted, culture densities were monitored at a wavelength of 600 nm (OD₆₀₀).

Plasmid Construction. Plasmids used in this study are listed in Table S2, Supporting Information. We constructed a new plasmid, termed pBAD30-Kan, which contains the P_{araBAD} promoter for regulated gene expression, a low-copy origin (p15A, 10–12 copies/cell), and the kanamycin resistance cassette. Briefly, this was done by ligating the gel-purified SphI–AhdI fragment of pBAD18-Kan with the gel-purified AhdI–SphI fragment of pBAD30. Ligation products were used to transform *E. coli* DH10B, and the resulting vector was verified by analytical restriction digestion and DNA sequencing (Quintara Biosciences).

Oligonucleotides used in this study are listed in Table S3, Supporting Information. Polymerase chain reaction (PCR) was used to append the restriction sites EcoRI, NotI, and SpeI to the 5′ end of the *gfpmut2* gene, and PacI and XbaI to the 3′ end of *gfpmut2*. PCR was performed with primers MF1 and MF2 and KOD DNA Polymerase (EMD Chemicals). The resulting PCR fragment and pBAD30-Kan were both digested with EcoRI and XbaI. The digestion products were gel purified and ligated, and the ligation products were used to transform *E. coli* DH10B. The designed insertion site of the resulting vector, pBAD30-Kan-*gfpmut2*, reads NotI(GCGGCCGC)-CTGCAASpeI(ACTAGT)-*gfpmut2*-PacI(TTAATTAA)-GCTACTXbaI(TCTAGA). The vector was verified by analytical restriction digestion and DNA sequencing.

The restriction sites NotI, SpeI, PacI, and XbaI do not occur within the *E. coli* *tolC*, *acrA*, and *acrB* genes, which code for the AcrAB-TolC efflux pump. PCR was used to generate three DNA fragments from *E. coli* JA300 (gift from Masaaki Wachi) genomic DNA prepared with the Qiagen Generation Column Capture Kit: One each for the *E. coli* *tolC* (MF3 and MF4), *acrA* (MF5 and MF6), and *acrB* (MF7 and MF8) genes. PCR was performed using KOD Xtreme Hot Start DNA Polymerase (EMD Chemicals). PCR was then used to append a 5′ NotI site, a C-terminal 6xHis tag, and a 3′ SpeI site to *tolC* (MF9 and MF10), a 5′ SpeI site, a C-terminal 6xHis tag, and a 3′ PacI site to *acrA* (MF11 and MF12), and a 5′ PacI site, a C-terminal 6xHis tag, and a 3′ XbaI site to *acrB* (MF13 and MF14). PCR was performed with KOD DNA polymerase (EMD Chemicals). The PCR products encoding either *tolC*, *acrA*, or *acrB* were gel purified and digested with NotI and SpeI, SpeI and PacI, and PacI and XbaI, respectively. The digestion products were purified with the Qiagen PCR Clean Up Kit.

pBAD30-Kan-*gfpmut2* was digested with NotI and SpeI. The vector backbone was gel purified and ligated with the NotI–SpeI *tolC* fragment and the ligation products were used to transform *E. coli* DH10B. The resulting vector, pBAD30-Kan-*tolC-gfpmut2*, was confirmed by analytical restriction digestion. pBAD30-Kan-*tolC-gfpmut2* was digested with SpeI and PacI. The vector backbone was gel purified and ligated with the SpeI–PacI *acrA* fragment and the ligation products were used to transform *E. coli* DH10B. The resulting vector, pBAD30-Kan-*tolC-acrA*, was confirmed by analytical restriction digestion. pBAD30-Kan-*tolC-acrA* was digested with PacI and XbaI. The vector backbone was gel purified and ligated with the PacI–XbaI *acrB* fragment; ligation products were used to transform *E. coli* DH10B. The resulting vector, pBAD30-Kan-*tolC-acrA-acrB*, was confirmed by analytical restriction digestion and DNA sequencing.

Library Construction. Whole Gene Random Mutagenesis Library. Whole-gene error-prone PCR (0.5 μ M MF15 and MF16; 1 $\times$ GoTaq Green Reaction Buffer; 0.2 mM dATP and dGTP; 1 mM dCTP and dTTP; 5.5 mM $MgCl_2$; and 5 units GoTaq DNA Polymerase (Promega)) was used to generate a pool of mutated *acrB* PCR products. The PCR products were gel purified, digested with *PacI* and *XbaI*, purified, and ligated with the likewise digested, gel-purified pBAD30-Kan-*tolC-acrA-acrB* backbone. The ligation products were electroporated into ElectroMAX DH10B-T1 (Invitrogen) or TransformMax EC100 *E. coli*. After growth for 1.25 h at 37 °C and 225 rpm, cells were spread on LB-agar-Kan plates. Approximately 30,000 colonies were collected in LB-Kan liquid media; these cells were resuspended, and the culture was grown for 1 h. Frozen stocks of library were prepared in 15% (v/v) glycerol.

CPEC-Generated Loop 1 Library. Error-prone PCR (0.2 mM dATP, dGTP, dCTP, and dTTP; 3 mM $MgCl_2$; 0.4 μ M MF17 and MF18; 0.15 mM 8-oxo-2'-deoxyguanosine-5'-triphosphate; 5 μ M 2'-deoxy-p-nucleoside-5'-triphosphate; 1 $\times$ KOD Buffer 1; 1 unit KOD Polymerase; 0.2 ng/ μ L template) was used to mutagenize the 567 bp region encoding loop 1 of *AcrB*. PCR was then used to amplify the mutagenized loop insert fragment (MF19 and MF20) and the vector backbone (MF21 and MF22). Circular polymerase extension cloning (CPEC)^{51,52} was used to combine the mutagenized loop insert fragment and vector backbone (0.2 mM dATP, dGTP, dCTP, and dTTP; 1 $\times$ Xtreme Buffer; 155 ng vector backbone; 12.6 ng mutagenized loop insert; 0.4 units KOD Xtreme Hot Start Polymerase). CPEC products were used to transform ElectroMAX DH10B competent cells (Invitrogen).

Combinatorial Site-Directed Mutagenesis of Loop 2 Residues. A 72-base oligonucleotide, MF24, was designed with NNK (any base/any base/G or T) codons at amino acid positions 610, 612, 615, and 617 and a 53-base oligonucleotide, MF25, was designed with NNK codons at amino acid positions 626 and 628. DNA polymerase I, large (Klenow) fragment (New England BioLabs) was used to generate double-stranded DNA of this portion of the *acrB* gene. This DNA segment encoded residues 602–635 of *AcrB*. The DNA segments encoding residues 1–601 (MF15 and MF23) and 636–1049 (MF26 and MF16) were generated by PCR. Overlap PCR was used to assemble the three fragments. The resulting mutagenized *acrB* was gel-purified, digested with *PacI* and *XbaI*, purified, and ligated with the likewise digested, gel-purified pBAD30-Kan-*tolC-acrA-acrB* backbone. Ligation products were used to transform TransformMax EC100 *E. coli* (Epicenter).

Exponential Growth Competition. One aliquot of the library was thawed and placed on ice; after mild vortexing, 50 μ L was aliquoted into 10 mL LB-Kan medium in a screw-cap test tube (Pyrex No. 9825/6). This culture was shaken for 30 min. The culture was then divided between two screw-cap test tubes. To one of the tubes, *n*-butanol was added to a final concentration of 0.5% (v/v). After 1 h of incubation, both cultures were diluted 1:100. Culture densities were monitored until reaching an OD₆₀₀ of 0.4, and then diluted 1:100. Serial dilutions were carried out twice more such that cultures were diluted 1:100 and grown to an OD₆₀₀ of 0.4 a total of three times. Some cells from each culture were then spread onto agar plates.

Single colonies were isolated and used to start overnight cultures. The cultures were diluted to an OD₆₀₀ of 0.05 in liquid

medium containing 0.5% *n*-butanol in screw-cap test tubes or microtiter plates.

Characterization of Selection output. For each strain that warranted further characterization and analysis, the plasmid DNA was purified and sent for DNA sequencing. The plasmids were also each used to transform *E. coli* DH10B and spread onto agar plates. Single colonies were used to start overnight cultures. The cultures were diluted to an OD₆₀₀ of 0.05 in medium with the appropriate amount of inhibitor in screw-cap test tubes. Three colonies were analyzed per strain. Culture density was monitored over time with a Genesys 20 spectrophotometer and compared to strains harboring empty vector and wild-type *acrB*. The inhibitors and concentrations used were 6% ethanol, 0.7% *n*-butanol, 0.8% isobutanol, 0.2% *n*-pentanol, 0.1% *n*-hexanol, 0.04% *n*-heptanol, 0.035% 3-octanol, 0.005%, 0.012%, and 0.023% *n*-octanol, and 2.1 mg/L chloramphenicol. All percentages are given as volume by volume (v/v).

Construction of Mutations in *acrB*. Oligonucleotides (MF28–MF35) were designed to use the Gibson assembly method for site directed mutagenesis.⁵³ Whole-vector PCR and Gibson assembly (Gibson Assembly Master Mix, New England BioLabs) was performed as described.

Microtiter Plate-Based Growth Assays. Overnight cultures were diluted to an OD₆₀₀ of 0.05 in 200 μ L medium per well containing the indicated concentration of inhibitor. The inhibitor *n*-butanol was used at 0.3%, 0.6%, 0.7%, and 0.8% (v/v), and ampicillin at 20 mg/L. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used at 50 μ M and 0.5% (w/v) glucose supplemented to the medium as indicated. Honeycomb microtiter plates (Growth Curves U.S.A.) were sealed with MicroAmp optical adhesive film (Applied Biosystems) and incubated in a Bioscreen-C Automated Growth Curve Analysis System (Growth Curves U.S.A.) with maximum continuous shaking.

End Point Growth Assays. Frozen stocks were streaked onto LB-Kan plates. Multiple colonies were used to inoculate 10 mL LB-Kan aliquots (3 unique samples per strain per condition) with either no *n*-butanol or supplemented with 0.33, 0.45, 0.55, or 0.66% *n*-butanol (v/v) in screw-cap test tubes. Cultures were incubated at 37 °C with shaking at 225 rpm for 14 h. Culture density was assayed with a Genesys 20 spectrophotometer. Culture densities were normalized to the culture density of the strain harboring empty vector.

Extracellular *n*-Butanol Measurements. Frozen cell cultures with appropriate variants of *AcrB* were streaked onto agar plates and grown overnight. Six colonies of wtAcrB and Var1-2 were all cultured in 2 mL LB-Kan for 10 h and then subcultured 1:500 into 100 mL growth medium to grow overnight (12–16 h). After growth, OD₆₀₀ measurements were taken and 100OD₆₀₀ of cells (approximately 40–50 mL of culture, based on OD₆₀₀ measurements) were centrifuged for 5 min at 4000g. The supernatant was discarded and cells were washed twice with Phosphate Buffered Saline (PBS pH 6.5). During the final wash, the cells were transferred to 1.5 mL microcentrifuge tubes, centrifuged, and the supernatant was carefully aspirated. The cells were then resuspended in 400 μ L PBS containing 0.6% *n*-butanol and more PBS + 0.6% *n*-butanol was added until the final volume was 1.0 mL. Samples were incubated at room temperature for 1 h and then centrifuged again. 200 μ L of the supernatant was taken for quantification by liquid chromatography (LC), which required mixing with 50 μ L PBS + 1.2% isopropanol as an internal

standard. The rest of the supernatant was aspirated, the cells were resuspended in total volume of 1 mL PBS, and final OD₆₀₀ readings were taken to normalize the butanol concentration.

Butanol concentration was calculated by HPLC. Ten μ L of the samples were injected onto an Rezex RFQ Fast Acid H+ (8%) (100 $\times$ 7.8 mm, Phenomenex, Torrance, CA) column and analyzed at 55 °C on a 1200 series liquid chromatography instrument equipped with a refractive index detector (Agilent Technologies, Santa Clara, CA). Elution was performed with 5 mM sulfuric acid at a flow rate of 1.0 mL/min.

The sequestered *n*-butanol concentration was calculated by subtracting the measured *n*-butanol concentration of the sample supernatant from the average of three measurements of a no-cell 0.6% *n*-butanol control. The difference was then normalized to 100 OD₆₀₀ based on the final OD₆₀₀ readings. Reported values represent six independent experiments.

Membrane Integrity Assays. Overnight cultures were diluted to an OD₆₀₀ of 0.05 in LB-Kan medium. Cultures were incubated in screw-cap test tubes for 5.5 h, allowing the strains to complete log phase growth. Samples were normalized by OD₆₀₀; culture medium supernatants, 10-fold trichloroacetic acid-concentrated culture medium supernatants,⁵⁴ and whole cell lysates were assayed for the presence of GroEL and maltose binding protein by Western blot.

Propidium iodide (PI) staining protocols were adapted from a previous study⁵⁵ with minor changes. Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.05 in 3 mL LB-Kan medium and incubated for 5–6 h until an OD₆₀₀ of 0.8 was reached. 0.5OD of cells were centrifuged for 5 min at 5000g and resuspended in PBS with the addition of 10 μ g/mL propidium iodide and 2.5 μ g/mL fluorescein isothiocyanate (FITC) and incubated at room temperature in the dark for 15 min. Prior to staining, heat-shocked cells were incubated in PBS at 80 °C for 10 min. Cells were then diluted to $\sim$ 1000 cell/ μ L and analyzed by flow cytometry. FITC does not stain live cells and cells with positive FITC signal were not included in calculating PI uptake.

Western Blotting. Western blotting was performed as previously described but with some changes.⁵⁶ Briefly, supernatants, 10-fold concentrated supernatants, and whole cell lysates were mixed with SDS loading buffer containing 2-mercaptoethanol (final concentration loading buffer, 1 $\times$). The samples were incubated in a heat block for 5 min at 95 °C and 10 μ L of each sample was run on 7.5% or 12.5% Tris-glycine gels. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked in 5% (w/v) nonfat dry milk/TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, to pH 7.5) at room temperature for 1 h. The membranes were incubated with primary antibodies diluted in 1% milk/TBST at room temperature overnight. The following primary antibodies were used: monoclonal mouse anti-maltose binding protein (Sigma), diluted 1:5000; polyclonal rabbit anti-GroEL (Sigma), diluted 1:10000; and monoclonal mouse THE anti-His (Genscript), diluted 1:2000.

After washing in TBST, the membranes were incubated with secondary antibodies diluted in 1% milk/TBST at room temperature for 1 h. The following secondary antibodies were used: stabilized peroxidase conjugated goat anti-mouse, H+L (Thermo Scientific), diluted 1:1000, and stabilized peroxidase conjugated goat anti-rabbit, H+L (Thermo Scientific), also diluted 1:1000. After washing, proteins were detected using the

SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and images captured with a ChemiDoc XRS+ imaging system (Bio-Rad).

■ ASSOCIATED CONTENT

● Supporting Information

Figures S1–S7: additional evidence supporting the conclusions of this paper, including growth curve assays, images of the AcrB structure, membrane integrity experiments, and Western blot analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

D.T.E. and M.A.F. conceived the project. M.A.F. made the pBAD30-Kan vector, cloned *acrAB-tolC*, constructed the *acrB* libraries and deletion strains, performed the selections, carried out growth curve assays, made site-directed mutants, and performed membrane permeability experiments. S. Boyarskiy performed the expression level, membrane permeability, and extracellular butanol concentration experiments. M.R.Y. and N.K. made site-directed mutants and carried out growth curve assays. S. Bauer assisted in the design and interpretation of *n*-butanol concentration quantitation experiments. D.T.E. assisted in the design and interpretation of all experiments. M.A.F., S. Boyarskiy, and D.T.E. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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