AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Guanidine Riboswitch-Regulated Efflux Transporters Protect Bacteria against Ionic Liquid Toxicity

Douglas A. Higgins,[a,b]* John M. Gladden,[a,c] Jeff A. Kimbrel,[b] Blake A. Simmons,[a,d] Steven W. Singer,[a,d] Michael P. Thelen[a,b]

[a]The Joint BioEnergy Institute, Emeryville, California, USA
[b]Lawrence Livermore National Laboratory, Livermore, California, USA
[c]Sandia National Laboratories, Livermore, California, USA
[d]Lawrence Berkeley National Laboratory, Berkeley, California, USA

ABSTRACT Plant cell walls contain a renewable, nearly limitless supply of sugar that could be used to support microbial production of commodity chemicals and biofuels. Imidazolium ionic liquid (IIL) solvents are among the best reagents for gaining access to the sugars in this otherwise recalcitrant biomass. However, the sugars from IIL-treated biomass are inevitably contaminated with residual IILs that inhibit growth in bacteria and yeast, blocking biochemical production by these organisms. IIL toxicity is, therefore, a critical roadblock in many industrial biosynthetic pathways. Although several IIL-tolerant (IIL^T) bacterial and yeast isolates have been identified in nature, few genetic mechanisms have been identified. In this study, we identified two IIL^T *Bacillus* isolates as well as a spontaneous IIL^T *Escherichia coli* lab strain that are tolerant to high levels of two widely used IILs. We demonstrate that all three IIL^T strains contain one or more pumps of the small multidrug resistance (SMR) family, and two of these strains contain mutations that affect an adjacent regulatory guanidine riboswitch. Furthermore, we show that the regulation of *E. coli sugE* by the guanidine II riboswitch can be exploited to promote IIL tolerance by the simple addition of guanidine to the medium. Our results demonstrate the critical role that transporter genes play in IIL tolerance in their native bacterial hosts. The study presented here is another step in engineering IIL tolerance into industrial strains toward overcoming this key gap in biofuels and industrial biochemical production processes.

IMPORTANCE This study identifies bacteria that are tolerant to ionic liquid solvents used in the production of biofuels and industrial biochemicals. For industrial microbiology, it is essential to find less-harmful reagents and microbes that are resistant to their cytotoxic effects. We identified a family of small multidrug resistance efflux transporters, which are responsible for the tolerance of these strains. We also found that this resistance can be caused by mutations in the sequences of guanidine-specific riboswitches that regulate these efflux pumps. Extending this knowledge, we demonstrated that guanidine itself can promote ionic liquid tolerance. Our findings will inform genetic engineering strategies that improve conversion of cellulosic sugars into biofuels and biochemicals in processes where low concentrations of ionic liquids surpass bacterial tolerance.

KEYWORDS *Bacillus*, biofuels, functional genomics, guanidine riboswitch, ionic liquids, quaternary ammonium compounds

A major goal of biotechnology is to convert carbon in plant biomass into biofuels and commodity chemicals using microbes. While some plants, such as sugarcane and corn, are accessible sources of sugar for this process, these sources compete with

Citation Higgins DA, Gladden JM, Kimbrel JA, Simmons BA, Singer SW, Thelen MP. 2019. Guanidine riboswitch-regulated efflux transporters protect bacteria against ionic liquid toxicity. J Bacteriol 201:e00069-19. https://doi.org/10.1128/JB.00069-19.

Editor Tina M. Henkin, Ohio State University

Address correspondence to Michael P. Thelen, mthelen@llnl.gov.

* Present address: Douglas A. Higgins, Pivot Bio, Berkeley, California, USA.

Received 22 January 2019
Accepted 9 April 2019
Accepted manuscript posted online 15 April 2019
Published 10 June 2019

the food supply, have poor life cycle profiles, and are not available on the level required to upset global petroleum markets. Agricultural waste and noncommercialized plants such as switchgrass are a largely untapped and abundant supply of biomass. The major component of dry plant biomass is cellulose, a polymer of glucose that is found in a semicrystalline form in plants. This glucose is inaccessible and must be extracted from cell walls using a chemical pretreatment that enables efficient enzymatic breakdown.

Biomass pretreatments include dilute acid hydrolysis, ammonia fiber expansion, and ionic liquid solubilization (1, 2). These treatments disrupt hydrogen bonds among cellulose, hemicellulose, and lignin, the dominant constituents of biomass. Following pretreatment, cellulose becomes accessible to enzymes (3–5) and can be further broken down to yield sugars for growing microbes that have been engineered to make biofuels or commodity chemicals.

Of the existing pretreatment strategies, imidazolium ionic liquids (IILs) uniquely solubilize cellulose in a manner agnostic to the type of biomass being broken down (6). Similarly, they alone separate lignin for independent valorization and avoid various breakdown products that result from pretreatment of different biomass. IILs, however, suffer from issues of cost as well as IIL-mediated inhibition of downstream fermentation. Ultimately, new recycling methods and development of less-expensive IILs are expected to reduce IIL cost, but the toxicity of quaternary ammonium compounds (QACs), a recurring feature even in many newer IILs, often persists to downstream fermenters (7, 8).

Accordingly, several environmental microbes have been identified that are tolerant to ionic liquids such as 1-ethyl-3-methylimidazolium acetate ($[C_2C_1im][OAc]$), 1-ethyl-3-methylimidazolium chloride ($[C_2C_1im]Cl$), or other closely related IILs. Specifically, environmental studies have identified one Gram-negative bacterium (*Pluralibacter* [previously *Enterobacter*] *lignolyticus*) (9, 10) and several Gram-positive bacteria (*Brevibacterium sanguinis*, *Rhodococcus erythropolis*, *Bacillus coagulans*, and *Bacillus amyloliquefaciens*) (11–13) and fungi (*Aspergillus*, *Saccharomyces cerevisiae*, and a number of non-*Saccharomyces* yeast strains) (14–16) that display various levels of tolerance to these IILs.

Even though several IIL-tolerant (IIL^T) strains have been identified, published work on the mechanisms of tolerance is limited. In previous research, transcriptional responses of the Gram-negative *P. lignolyticus* strain were analyzed, and specific genes that protect biofuel-producing *Escherichia coli* from IILs were identified (10, 17). Two of these genes form a genetic cassette encoding a multidrug efflux pump of the major facilitator superfamily (MFS) and an autoregulatory component, a TetR-type repressor of the MFS gene (*eilA* and *eilR*, respectively). Moreover, in an environmental strain of the yeast *Saccharomyces cerevisiae*, we discovered that tolerance to IILs can be attributed to two single nucleotide polymorphisms (SNPs) in the MFS efflux pump encoded by *SGE1* (16). Although *SGE1* is known to protect yeast against QACs, such as crystal violet (18), that share some chemical features with imidazolium cations, laboratory strains and many environmental isolates of *S. cerevisiae* are highly IIL sensitive (IIL^S). Just as the *eilA* gene can grant IIL tolerance to *E. coli* (17), introduction of the environmental *SGE1* variant confers tolerance to the IIL^S lab strain of *S. cerevisiae* (16). We also identified a second yeast IIL tolerance gene, *ILT1*, which encodes an uncharacterized membrane protein (16). Apart from these natural isolates, adaptive lab evolution was recently used to generate IIL^T mutants of *E. coli*. The resulting mutations are in *mdtJI* and *yhdP*, encoding a multidrug efflux pump and an uncharacterized transporter, respectively (19), and in *cydC*, encoding a cytochrome assembly factor that is a component of an ABC transporter complex (20). From these few reports, we believe that the toxic effects of IILs are reduced by membrane proteins that export $[C_2C_1im]^+$ cations from cells.

In this investigation, we set out to identify whether other tolerance mechanisms exist in bacteria that would enable *E. coli* to successfully produce biofuels in the presence of IILs. We expected to find MFS transporter genes in IIL^T environmental bacteria, but instead we discovered several small multidrug resistance (*smr*) genes that

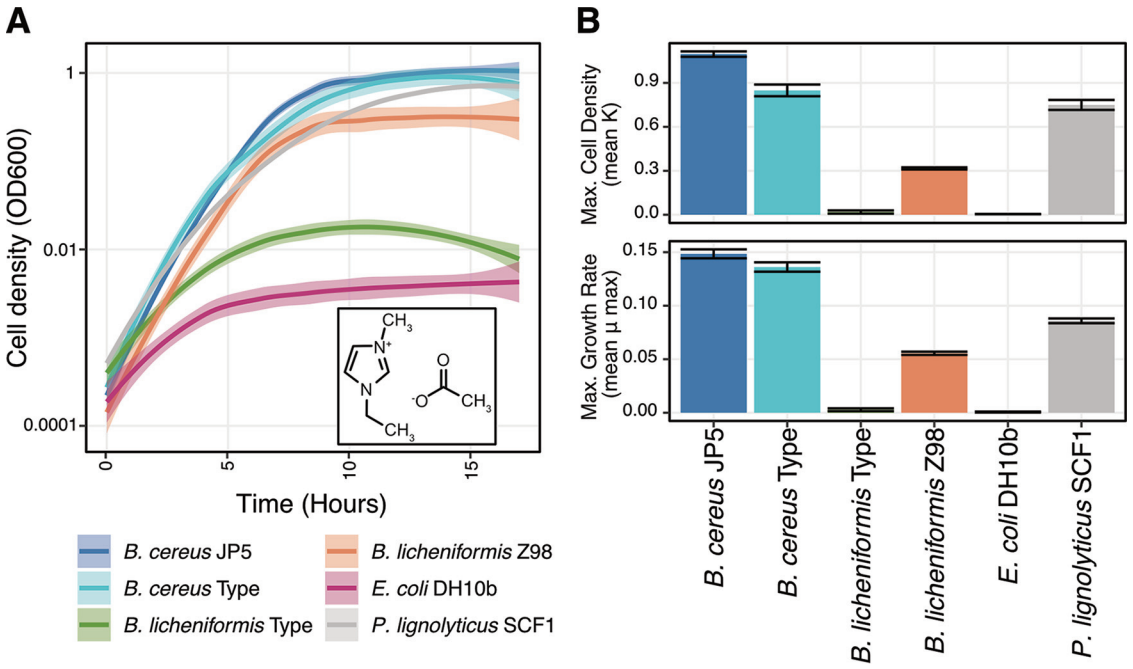

FIG 1 IIL tolerance of selected bacterial strains. Growth curves (A) and maximum growth rate and maximum cell density (B) are shown for each strain cultured in IIL medium (250 mM [C_2C_1im][OAc]). The chemical structure of [C_2C_1im][OAc] is shown in the inset in panel A.

confer a similar tolerance phenotype in isolates of *Bacillus* and *E. coli*. The *Bacillus smr* genes promote full IIL tolerance functionality in *E. coli*. Of particular interest, we found that guanidine riboswitch mutations (21, 22) lead to SMR pump expression and IIL tolerance; in the absence of these mutations, addition of guanidine itself to *E. coli* results in tolerance.

RESULTS

Very-IIL-tolerant organisms. To expand our pool of IIL^T organisms, we isolated bacteria from two green waste composting facilities in northern California and screened them for growth on a medium containing 294 mM (5%, wt/vol) [C_2C_1im][OAc]. We found several bacterial strains that reproducibly grew well in the presence of this IIL and isolated six of these for further testing. Four of the isolates were moderately IIL^T and were identified by 16S rRNA gene sequencing as *Bacillus subtilis* strains. One isolate, identified as a *Bacillus cereus* strain (*B. cereus* JP5), displayed tolerance to 250 mM [C_2C_1im][OAc] (Fig. 1A). This strain could even produce large colonies overnight on 500 mM [C_2C_1im][OAc], representing a much greater bacterial tolerance to this industrially relevant IIL than that of other bacterial and yeast strains that we have isolated. A *Bacillus licheniformis* strain (*B. licheniformis* Z98) was also IIL tolerant, with a maximum growth rate and final cell density of about one-third of those of the *B. cereus* type and JP5 strains, while our previously characterized IIL-tolerant *P. lignolyticus* isolate (10) grew at about half the rate (Fig. 1B). Due to their high-tolerance phenotypes, we concentrated our studies on the *B. cereus* and *B. licheniformis* isolates.

***B. cereus smr* genes protect *E. coli*.** Functional genomics is a powerful technique that we have recently used specifically to identify IIL tolerance mechanisms (16, 17). In this approach, large donor DNA regions (~35 kb) from the tolerant organism are delivered to *E. coli* or *S. cerevisiae* by fosmid cloning, and cells are then immediately screened for their ability to grow on IIL-containing media. We chose to employ this technique using our most tolerant isolate, *B. cereus* JP5, as our DNA donor strain. After creating a fosmid library covering the *B. cereus* JP5 genome, we screened ~4,750 *E. coli* clones for tolerance to both 125 mM [C_2C_1im][OAc] and 136 mM [C_2C_1im]Cl. We have

previously seen that different mechanisms account for tolerance to the acetate anion and the more toxic $[C_2C_1im]^+$ cation of $[C_2C_1im][OAc]$ (10, 17). Here, we observed that no *E. coli* colonies developed on the $[C_2C_1im][OAc]$ medium. However, 56 IIL^T isolates grew well on the $[C_2C_1im]Cl$ medium; 12 were selected for further analysis and sequencing.

By end sequencing the positive fosmid clones and mapping them to the closely related *B. cereus* 10987 genome, we found that they cluster into 2 different regions (Fig. 2A). Fosmids from 5 isolates cluster in one overlapping region, region 1, sharing a core 27 kb of DNA (bp 924404 to 951379 of the 5,224,283-bp *B. cereus* 10987 genome). The remaining 7 fosmids cluster in region 2, sharing a core 12 kb of DNA (bp 3987520 to 3998586 of the same genome). While any given fosmid contained about ~40 kb of genomic DNA, we reasoned that the causative agents of tolerance occurred in the intersects of the two regions upon which our fosmid libraries converged.

We focused first on the smaller region, region 2, which contains 13 annotated genes and an additional 6 genes encoding hypothetical proteins (Fig. 2A). Because we had previously identified a *P. lignolyticus* MFS efflux pump which confers IIL tolerance to *E. coli* (17), we examined region 2 first for MFS pumps. No MFS pumps are present in this region, but we did notice members of two other transporter families. We found an ABC peptide transporter as well as a pair of small multidrug resistance (SMR) family-type pump genes, both of which were annotated as homologs of *sugE* (23) from *E. coli*. The *sugE*-like gene caught our attention because related SMR pumps are known to export QACs. Specifically, the *B. subtilis* SMR operon *gdnCD* (synonymous with *ykkCD*) encodes a dual efflux pump that, when expressed in *E. coli*, confers resistance to the guanidinium cation as well as to a range of other QACs, including methyl viologen, proflavine, and cetylpyridinium chloride (21, 24). The MFS pump EilA from *P. lignolyticus* protects *E. coli* from $[C_2C_1im]^+$ as well as a similar list of QACs (17). Due to the overlapping substrate preferences for these two pumps, we reasoned that this SMR pump might be the key factor in the protective activities of the cloned fosmids. Accordingly, we cloned the SMR pump genes from *B. cereus* JP5 into a low-copy-number vector in which the pump genes were driven, as on the fosmids, by their endogenous promoters. This plasmid protected *E. coli* better than the corresponding fosmid clones, resulting in about twice the final cell density (Fig. 2B).

The 27-kb region 1 covered by the remaining 6 fosmids contains 33 genes, and two of these also encode a SugE-like paired SMR pump, the only other such pairing in the *B. cereus* genome. When we cloned these SMR pump genes from *B. cereus* JP5 in a low-copy vector driven by the native promoter, we found, like for the SMR pumps in region 2, that they protected *E. coli* as well as or better than the fosmids that contained them (Fig. 2B).

Because we saw similar IIL tolerance exhibited by other bacterial members of the *B. cereus* group, specifically the type strains *B. cereus* and *B. thuringiensis* (Fig. 1 and 3), we tested the related SMR pumps from these organisms in *E. coli* for protection against $[C_2C_1im]^+$ toxicity. We observed that the pump pairs from the *B. thuringiensis* type strain protected *E. coli* in precisely the same way as the corresponding pumps from *B. cereus* JP5. These genes, here described as $pair1_{Bt}$ and $pair2_{Bt}$, encode proteins with accession numbers EEM67587 and EEM67588 (pair1) and EEM64372 and EEM64373 (pair2).

One SMR pump is a determining factor in IIL tolerance of the *B. cereus* group. Having demonstrated that SMR pumps from *B. cereus* JP5 or the *B. thuringiensis* type strain confer IIL tolerance to *E. coli*, we reasoned that these pumps are responsible for IIL tolerance in their native organisms. To test this, we focused our efforts on the *B. thuringiensis* type strain, as it was more readily genetically tractable than *B. cereus* JP5 and displays IIL tolerance similar to that of that organism (Fig. 3).

We created genetic deletions (25) of the *B. thuringiensis* type strain SMR transporter genes, $pair1_{Bt}$ and $pair2_{Bt}$. When contrasted with the wild-type parent in $[C_2C_1im][OAc]$, the $\Delta pair1_{Bt}$ strain had no identifiable phenotype (Fig. 3). The $\Delta pair2_{Bt}$ strain, however, showed no measurable growth under these conditions. To demonstrate that the $\Delta pair2_{Bt}$ deletion was responsible for the observed pheno-

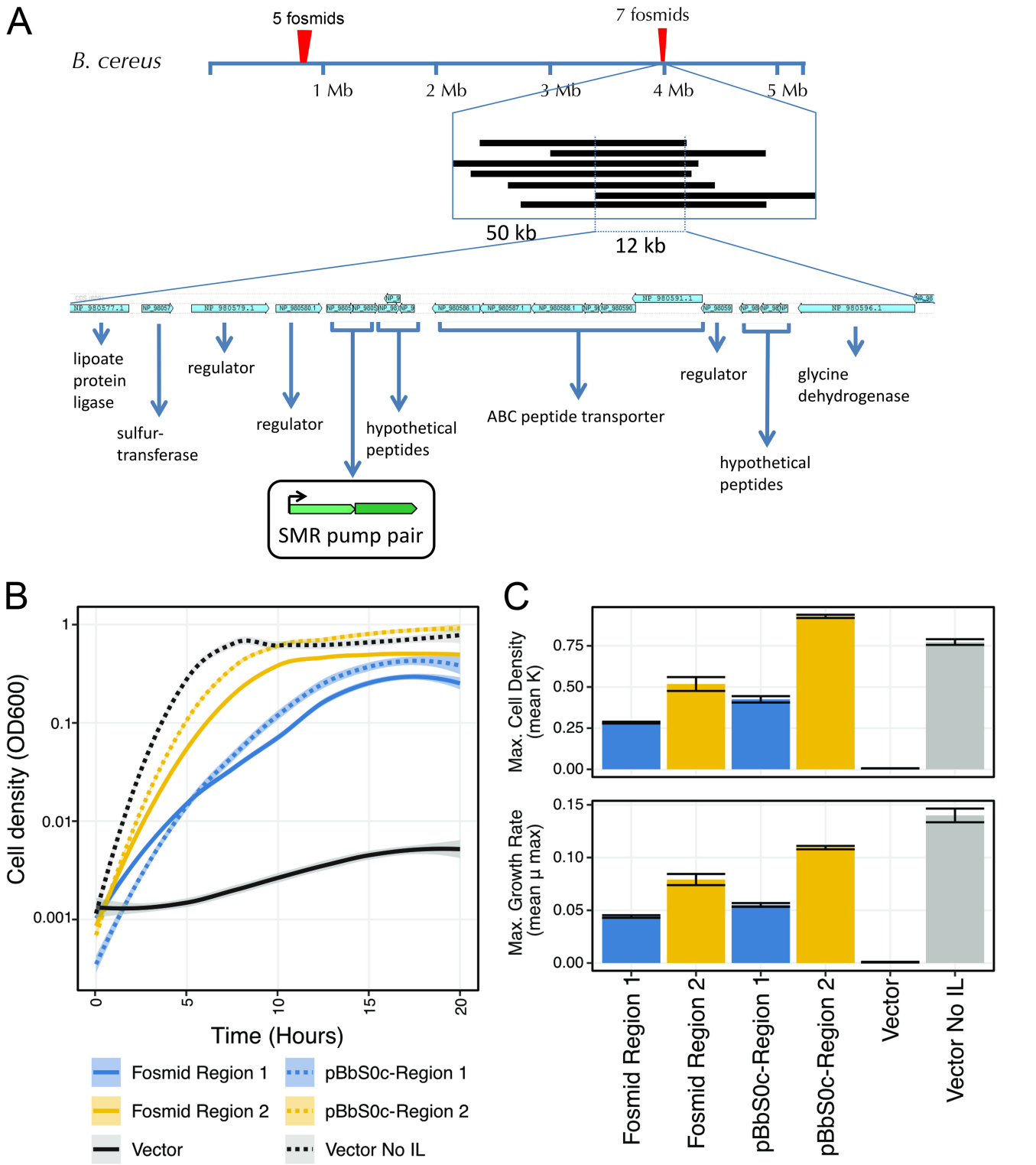

FIG 2 Identification of genome regions containing IIL tolerance genes. (A) The top blue line depicts the *B. cereus* loci from which the IIL^T-fosmid inserts originated. Region 2 is expanded below. (B and C) As indicated by growth curves (B) and maximum growth rate and maximum cell density (C), *E. coli* is protected from IIL (250 mM [C_2C_1im]Cl) by both fosmids and pBbS0c plasmids (48) containing *B. cereus* DNA from these loci.

type, we cloned the *pair2*$_{Bt}$ genes and native promoter into the low-copy-number (~4 copies) *B. thuringiensis* vector pHT304 (26). We observed that restoration of the transporter largely complemented the deletion (see Fig. S1 in the supplemental material). Because bacteria are very sensitive to the level of pump expression (17),

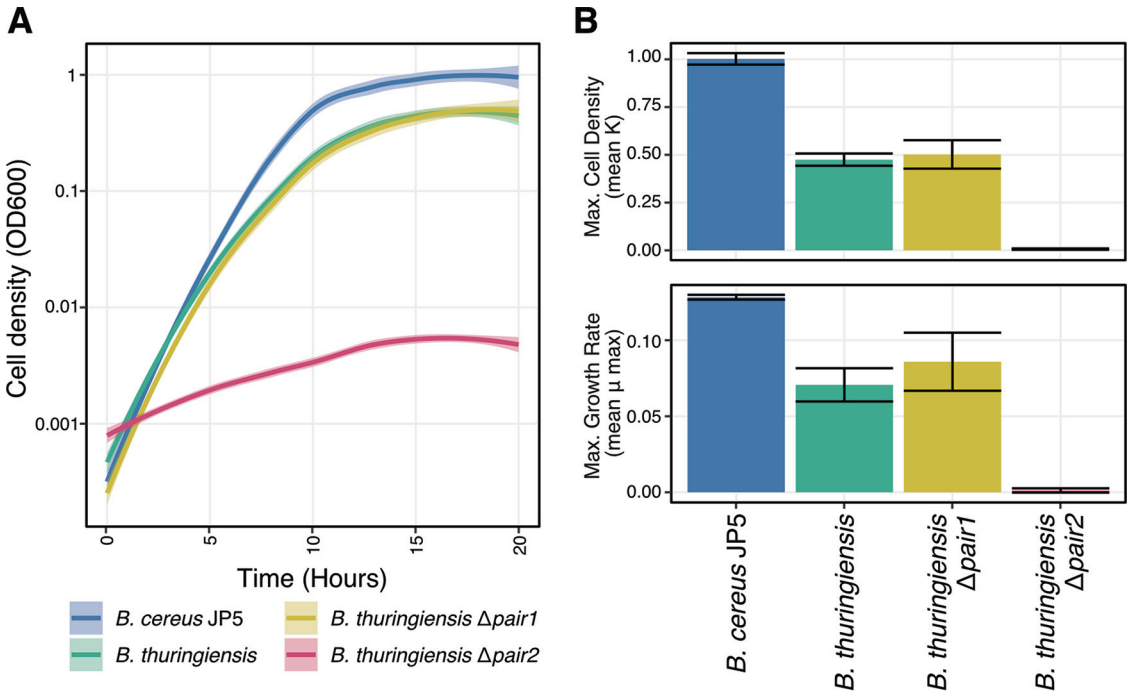

FIG 3 IIL tolerance in *B. thuringiensis* depends upon tandem SMR pump genes. Growth curves (A) and maximum growth rate and maximum cell density (B) are shown for each strain cultured in IIL medium (360 mM $[C_2C_1im][OAc]$).

it is possible that variable or perhaps unexpectedly high levels of efflux pumps were interfering with complete complementation.

A mutation in a guanidine riboswitch leads to IIL tolerance in *B. licheniformis*. Having demonstrated that SMR pumps in the *B. cereus* group are major IIL protection factors, we turned our attention to the *B. licheniformis* Z98 isolate. While all 3 tested members of the *B. cereus* group are very IIL tolerant, the tolerance phenotype of *B. licheniformis* Z98 is in marked contrast to that of the *B. licheniformis* type strain, which is highly IIL sensitive (Fig. 1). *B. licheniformis* is a close relative of the model Gram-positive organism *B. subtilis*, which has an intermediate IIL tolerance phenotype. *B. licheniformis* and *B. subtilis*, like *B. cereus*, each contain two pairs of SugE-like SMR pumps. To test these pump pairs individually, both the *gdnCD* and *yvdSR* genes along with their promoters were cloned from the tolerant *B. licheniformis* Z98 into plasmids and introduced into *E. coli*. We saw that both were proficient at protecting *E. coli* from IILs (see Fig. S2 in the supplemental material), regardless of which organism they originated from.

The GdnC and GdnD proteins are homologous to the *B. subtilis* proteins of the same name (74 and 72% amino acid identity, respectively). *B. subtilis* GdnCD ($GdnCD_{Bs}$), as mentioned above, is capable of protecting *E. coli* from QACs (24). *B. subtilis gdnCD* is preceded by the 5′ untranslated region (5′-UTR), now known as a guanidine riboswitch (21), that is found in many bacterial species, often before pump genes (21, 27–30). This riboswitch is predicted to contain 3 stem-loops in the first 155 nucleotides (nt), with the third stem-loop acting as an intrinsic transcription terminator: a strong stem followed by several U residues. Mutations in the third stem-loop can lead to pronounced upregulation of transcription of this gene by allowing transcriptional read-through (28).

When we examined the *yvdSR* and *gdnCD* regions in the IIL^S *B. licheniformis* type strain and in the IIL^T *B. licheniformis* Z98 isolate, we saw that the two strains had identical sequences in both regions except for one critical difference. Specifically, the 5′-UTR before *gdnCD* in the tolerant isolate is missing the last 26 nt in its predicted intrinsic transcriptional terminator. The nucleotides missing are the 26 that fall immediately before the run of U residues (UUUUUUCUUUU) that follows the terminator

stem-loop. This mutation closely parallels the mutation that leads to upregulation of *B. subtilis gdnCD* as mentioned above and is a result of inactivating the guanidine riboswitch (28).

To test if this change in the predicted transcriptional terminator stem-loop of *B. licheniformis gdnCD* ($gdnCD_{Bl}$) affected gene expression, we prepared transcriptional fusions. We cloned *gdnCD* from the tolerant Z98 isolate (26-nt terminator deletion) as well as from the type strain (wild-type terminator stem-loop) into pGFPamy and tested our constructs' abilities to confer tolerance to *Bacillus subtilis*, a close relative of *B. licheniformis* (31). When integrated into the genome of *B. subtilis*, the construct from the tolerant strain produced abundant green fluorescent protein (GFP) fluorescence, while the clone from the sensitive strain produced none that was detectable (see Fig. S3 in the supplemental material). We assessed the tolerance phenotypes of these same integrants and saw that *B. subtilis* carrying the Z98 *gdnCD* riboswitch leader was able to grow in 250 mM [C_2C_1im][OAc] medium, while *B. subtilis* carrying the 5′-UTR from the type strain was not (Fig. S3). Together, these experiments indicated that the loss of the 5′-UTR terminator stem-loop of $gdnCD_{Bl}$ increased gene transcription and boosted IIL tolerance. We propose that the same effect was occurring in the Z98 isolate and explains its pronounced IIL tolerance relative to that of the type strain (Fig. 1).

Being curious whether the 5′-UTR terminator stem-loop of $gdnCD_{Bl}$ consistently increased gene transcription in distantly related organisms, we tested the same pGFPamy plasmids in *E. coli*. The pGFPamy shuttle vector acts as an integration vector in Gram-positive bacteria but is maintained as a free plasmid in *E. coli* owing to its (high-copy ColE1) Gram-negative origin of replication. Consistent with our *Bacillus* results, we found that *E. coli* carrying pGFPamy-$gdnC_{Bl\text{-}Z98}$ produced far higher fluorescence than *E. coli* carrying $gdnCD_{Bl\text{-}Type}$ (data not shown). However, in contrast to our *Bacillus* results, we saw that low-level leaky pump expression from wild-type $gdnCD_{Bl}$ is sufficient for protecting *E. coli* from IILs (Fig. S2). We suggest that this difference is due largely to plasmid copy number.

A mutation in the *E. coli* guanidine II riboswitch is responsible for IIL tolerance. We expanded our identification of bacterial IIL tolerance mechanisms by screening a metagenomic library constructed from DNA isolated from Brazilian rainforest soils (32). From this screen, we selected four clones in *E. coli* that displayed IIL tolerance. Fosmid sequences that were correlated with high tolerance most closely aligned to genomes of the genus *Bradyrhizobium* and the phylum *Planctomycetes*. Two others with lower tolerance contained unique, nonoverlapping DNA from the genus *Bradyrhizobium*. However, when we transformed the four fosmids back into the parent *E. coli* strain, we were unable to recapitulate their tolerance phenotypes. Furthermore, when the positive *E. coli* clones from the screen were passaged until they lost their fosmids, these strains retained their tolerance phenotypes (see Fig. S4 in the supplemental material). We reasoned that the phenotype was clearly not linked to the fosmids but instead depended on one or more mutations which had occurred in the *E. coli* genome. As our previous experiments had demonstrated that *sugE*-like SMR genes were capable of conferring [C_2C_1im]$^+$ tolerance, we sequenced a 1.8-kb region surrounding the *E. coli* gene *sugE* and a 2.6-kb region surrounding the other SMR gene present in *E. coli*, *emrE* (33). No changes in the *emrE* sequence were found relative to the parent *E. coli* strain. We did, however, identify a single-base-pair transversion in the 5′-UTR of *sugE* for two highly tolerant strains (G to C in DH242 and G to T in DH244). We saw no changes in either region for the strains with lesser tolerance.

Until recently, a mechanism for *sugE* induction was not known. However, the *sugE* 5′-UTR contains the mini-*ykkC* motif, which was discovered to be an unusually short riboswitch that binds to the guanidinium cation (21, 22) and initiates the expression of SugE and similar transporters and permeases in other bacteria. More closely defining this as the guanidine II riboswitch (22, 34, 35), it contains two tandem hairpins (P1 and P2) with covariant stems and highly conserved loops containing an ACGR motif. Upon binding of guanidinium in the ACGR pocket, the conserved purines in the last position of both P1 and P2 are involved in a stacking interaction that could stabilize P1-P2

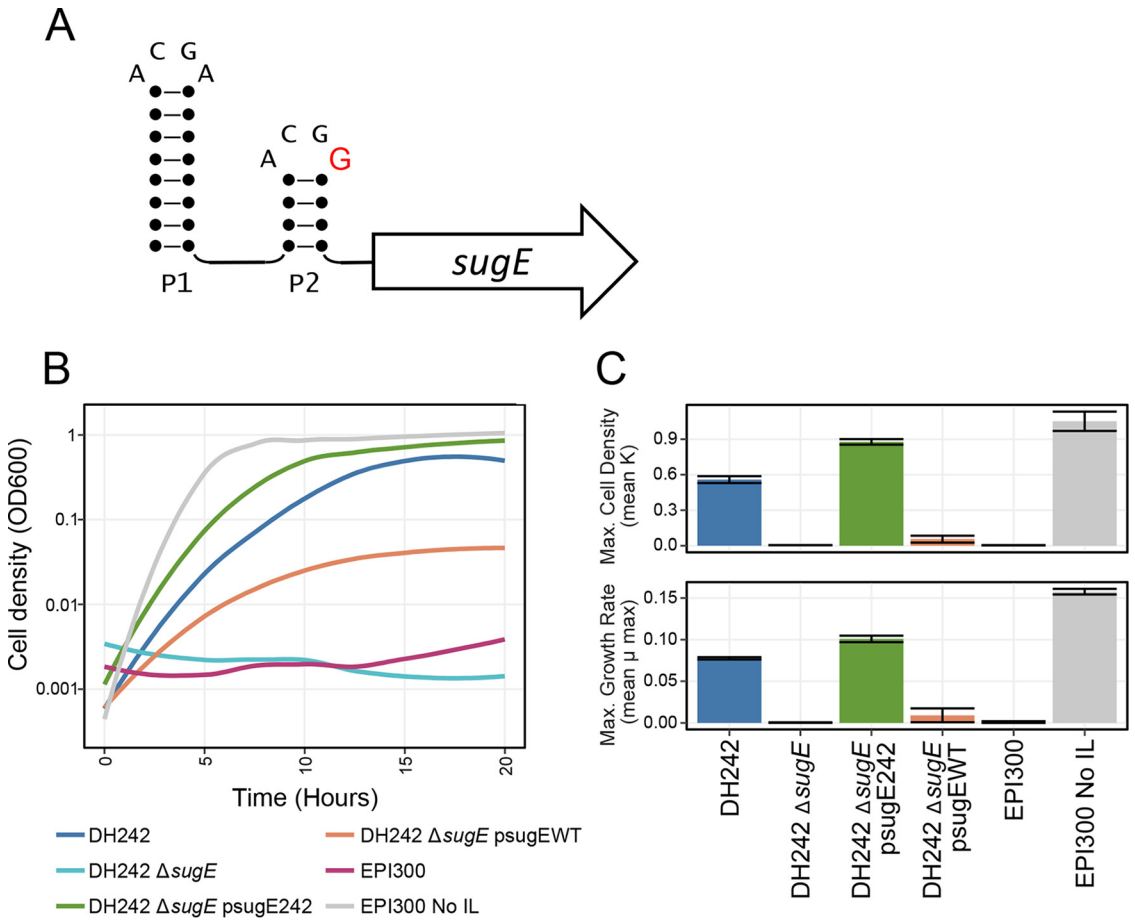

FIG 4 Point mutations in the guanidine II riboswitch confer IIL tolerance to *E. coli*. (A) The highly conserved ACGR sequence within the P1 and P2 hairpins of the guanidine II riboswitch that regulates *sugE* in *E. coli*. (B and C) Growth curves (B) and maximum growth rate and maximum cell density (C) are shown for the plasmid expression of various *sugE* constructs that complement Δ*sugE* in *E. coli* grown in IIL medium (250 mM $[C_2C_1im]Cl$).

stem-loop dimerization, a conformation that likely exposes the ribosomal binding site and activates translation (35). In wild-type IIL^S *E. coli*, the first of these sequences is an expected ACGA and the second is ACG**G** (Fig. 4A). The transversions in the *E. coli* strains occur in this second stem-loop, which becomes ACG**C** in the IIL^T strains DH242 and ACG**T** in DH244. Considering that conserved purine residue stacking in the stem-loops may stabilize the P1-P2 interaction, we would expect a transversion mutation to decrease loop dimerization and ultimately lower gene expression, increasing QAC sensitivity. However, our results described above suggest that the opposite phenotype is observed, i.e., that our mutants are instead more tolerant.

To determine if these transversions were responsible for the tolerance phenotype, we first deleted the *sugE* gene from DH242, DH244, and the parent *E. coli* EPI300 strain. We found that this mutation obliterated the tolerance phenotype in these strains (Fig. 4B). This proved that *sugE* was necessary for tolerance but did not yet prove that the transversion was sufficient for tolerance. We next cloned the *sugE* region either with or without the *sugE* riboswitch transversions into a low-copy-number plasmid to test for complementation of the *sugE* deletion. The results indicated that a plasmid containing the wild-type region sequence increased tolerance somewhat, presumably due to the gene being carried on a plasmid and thus slightly more than single copy. However, based on both the maximum growth rate and cell density measurements, the plasmid containing the region with the riboswitch transversion increased tolerance to up to 50% higher than that seen in our original mutant, DH242 (Fig. 4C). This enhanced tolerance may be due to the plasmid copy number. The *sugE* complementation

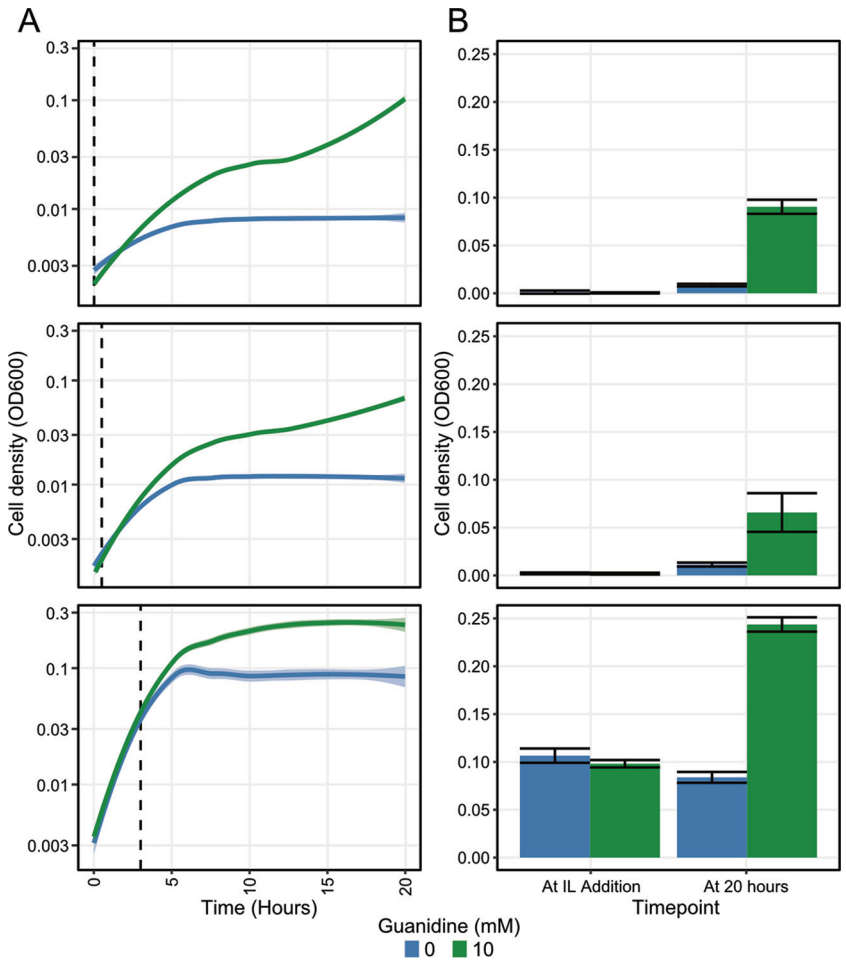

FIG 5 Addition of guanidine protects *E. coli* from IIL toxicity. *E. coli* DH10b was cultured in medium containing either 0 or 10 mM guanidine-HCl; 250 mM [C_2C_1im][OAc] was added at 0, 30, and 180 min after the start of culture. (A) Growth curves, with the addition of IIL indicated by dashed lines; (B) maximum cell density at the initial IIL addition and after 20 h of cultivation.

plasmids had the same effects in an EPI300 Δ*sugE* background as in a DH242 Δ*sugE* or DH244 Δ*sugE* background, demonstrating that this transversion is necessary and sufficient to explain the phenotype (see Fig. S5 in the supplemental material).

Guanidine activation of the *E. coli* riboswitch promotes IIL tolerance. Because the mutations in the two IIL^T *E. coli* strains are in guanidine-responsive riboswitches, we considered whether the wild-type guanidine II riboswitch could influence IIL tolerance in response to binding guanidine. We tested this by including guanidine in the culture medium of an IIL^S lab strain of *E. coli* and then adding [C_2C_1im][OAc] at different times. The results indicate that guanidine affords protection against IIL, as shown in Fig. 5 This was evident even when guanidine was added along with the IIL at the beginning of culture, but there was a more profound effect if cells were permitted to grow in the presence of guanidine for several hours before the IIL is added (Fig. 5, lower panels). In this case, cells reached an exponential growth rate until the IIL was added, and then cultures lacking guanidine were immediately and completely inhibited while cultures containing guanidine continued to grow. Suppression of growth beyond a cell density of about an optical density at 600 nm (OD_{600}) of 0.25 is caused by the toxic effects of the acetate anion, as noted previously (10, 17). This strategy potentially could be used to counteract the toxic effects of IILs in large-scale microbial production of biofuels or biochemicals, especially considering that guanidine salts are relatively inexpensive and that *E. coli* production strains are IIL^S.

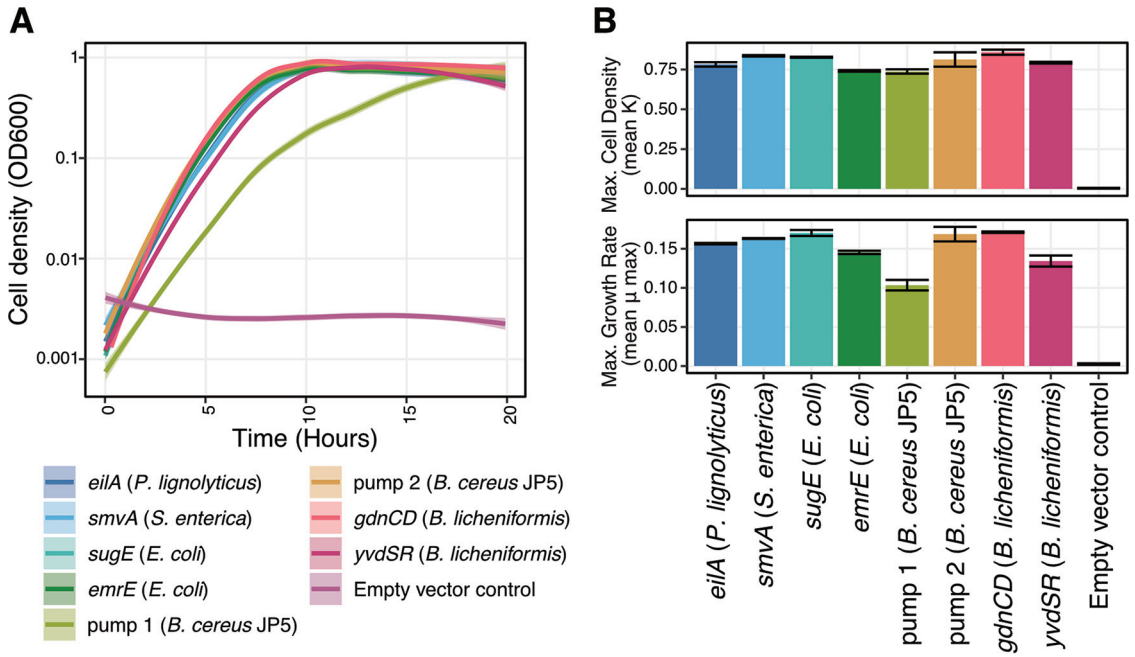

FIG 6 Efflux pump library tested in *E. coli*. Efflux pumps from the various organisms noted were expressed in *E. coli* DH10b in the IPTG-inducible vector pBbS6k. Growth curves (A) and maximum growth rate and maximum cell density (B) are shown for each strain cultured in medium containing 250 mM $[C_2C_1im]Cl$, 0.1 mM IPTG, and 50 mg/ml kanamycin.

A library of IIL efflux pumps. Our goal at the outset of this investigation was to identify new ionic liquid tolerance mechanisms beyond the MFS transporters we previously identified (16, 17). Our results include five SMR pumps, two from the *B. cereus* group, two from *B. licheniformis* and one from *E. coli*, that protected *E. coli* from the IILs tested. When expressed from their endogenous promoters, one *B. cereus* pump was reproducibly superior to the other (Fig. 1). Similarly, the *B. licheniformis* GdnCD pump appeared to be superior to the YvdSR pump (Fig. S2). To compare all pumps on an equal footing and separate pump activity from promoter strength, we cloned the previously identified MFS pump gene *eilA* (17), its homolog *smvA* from *Salmonella enterica* (36), the 5 SMR pump genes discussed here, and the SMR pump gene *emrE* (33) from *E. coli* and placed all of them after the same inducible promoter. We found that all but one transporter, the *B. cereus* region 1 transporter, appeared to be of equal strength (Fig. 6).

DISCUSSION

The power of IILs to effectively dissolve biomass, regardless of feedstock, and separate lignin for independent valorization makes them a keen area of study. Some of the most effective IILs, however, present downstream microbial toxicity issues that must be overcome. In previous work, we identified the *Pluralibacter* MFS-type transporter EilA, which relieves IIL toxicity and partly restores biofuel production in *E. coli* (17). In the study presented here, we show that several members of another family of transporters, SMR-type transporters, can relieve IIL toxicity in *E. coli* strains.

Although several IIL^T bacteria, especially from the genus *Bacillus*, have been identified, our study identified specific IIL tolerance factors from *Bacillus* and uniquely proved that these factors are necessary for tolerance in their native organisms. Understanding the precise native tolerance mechanisms is important for demonstrating that we are targeting major modes of tolerance as well as for analyzing tolerant organisms as potential production strains. *Bacillus* strains are the source of a number of secreted enzymes, such as proteases in detergents as well as amylases for starch breakdown (37). *Bacillus* spp. are also studied for their ability to grow on biomass and for their ability to produce enzymes and biochemicals for industry (38–40). Therefore, analysis of specific

methods of ionic liquid tolerance in *Bacillus* is not only useful for finding ways to bolster *E. coli* but also relevant for their own use in industry.

The IIL-protecting SMR-type transporters identified in this study come from Gram-positive *Bacillus* strains (two from the *B. cereus* group and two from *B. licheniformis*) or from *E. coli* (EmrE and SugE). Finding several transporters from such distantly related bacteria suggests that the groundwork already may be laid for identifying specific protein residues or motifs involved in IIL tolerance. That one of the *B. cereus* group pumps is inferior to the rest (Fig. 6) gives some opening for such studies. An array of pumps with variable IIL protection abilities would be needed for protein sequence alignments to be informative. Problematically, QAC transporters tend to have fairly broad specificity (41). This is known to be the case for *B. subtilis* GdnCD, which is homologous to *B. licheniformis* GdnCD investigated here, as well as several MFS- and SMR-type transporters. (*B. subtilis* GdnCD, like its *B. licheniformis* homolog, also protects *E. coli* from IILs [data not shown].) Due to this promiscuity, it is possible that such determinant factors do not exist for most SMR pumps.

In contrast, the *E. coli* SugE transporter is not a broad QAC transporter (23, 42). Most SMR transporters confer tolerance to some or all of the following compounds: benzyldimethyl tetradecylammonium chloride, benzalkonium chloride, pyronine Y, crystal violet, and ethidium bromide. SugE, however, protects from none of those and has been shown to transport only a narrow band of ammonium compounds featuring a 16-carbon chain: cetylpyridinium, cetyldimethylethyl, and hexadecyltrimethyl ammonium cations (23), in addition to guanidine (21, 22, 42). In the current study, the SugE protection repertoire has been expanded to include the small-chain $[C_2C_1im]^+$ cation. As *sugE* is native to *E. coli* and appears to be the most specific of the pump genes studied, it is likely to be particularly useful for future studies.

Moreover, *sugE* could be important for human health issues. A recent study tracking the five chromosomal QAC genes of *E. coli* as well as five that are frequently plasmid carried found that of these ten genes, *qacEΔ1* and *sugE*, when present on plasmids, were the best indicators of disinfectant resistance in retail meats (43). Similarly, a plasmid with a four-gene resistance cassette containing a β-lactamase gene as well as *sugE* was found to increase resistance in *Salmonella* and uropathogenic *E. coli* (44, 45). These studies suggest that *sugE* may be more important than previously recognized.

Our study highlights two situations where changes in a riboswitch leader affect the expression of SMR transporters. The mutation in the guanidine riboswitch that leads into *gdnCD* in *B. licheniformis* Z98, relative to the type strain of the same organism, is clearly a natural example of the designed mutation (M4) in the 5′-UTR of *B. subtilis ykkC* (*gdnC*) (28). Similar to M4, the Z98 deletion removes almost the entire stem-loop preceding the intrinsic transcriptional terminator. Inhibition of stem-loop formation is predicted to allow for enhanced GdnCD expression.

The unexpected tolerance phenotype of strains carrying a transversion mutation in the *E. coli sugE* guanidine II riboswitch P2 stem-loop has implications for our understanding of this regulatory element's mechanism. Crystal structures of guanidinium ligand-bound guanidine II riboswitch stem-loops suggest that ligand binding to the loop nucleotides stabilizes interloop base pairing among the two middle nucleotides of the P1 and P2 stem-loops (34, 35). This base pairing is believed to lead to changes in mRNA secondary structure that expose a ribosomal binding site and result in expression of the encoded protein. The fourth nucleotide of each loop, the site of our transversion mutations, has been proposed to potentially stabilize this dimerization through purine ring stacking and to have a positive effect on loop dimerization. We might, consequently, expect a transversion mutation to either decrease dimerization and tolerance protein expression or have no effect at all. However, we saw the opposite tolerance phenotype. It is intriguing that these residues are conserved purines and that substitution with either pyrimidine in *E. coli* increases IIL tolerance. The fact that either transversion alters the phenotype suggests that this change is not due to new Watson-Crick base-pairing interactions. Perhaps the bulky purine residues in the wild-type sequence inhibit a more favorable, constitutive loop dimerization interaction. Alterna-

tively, pyrimidine residues in the mutant strains may actively stabilize loop dimerization through an unknown mechanism or destabilize the interactions that are believed to block ribosomes from accessing the *sugE* ribosomal binding site. Another possibility, although less likely, is that the mutation leads to binding of the imidazolium cation, stabilizing the stem-loop dimerization and activating efflux pump expression.

Having identified guanidine II riboswitch mutants that lead to SugE protein expression phenotypes (i.e., IIL tolerance), we were curious to see if we could induce IIL tolerance in wild-type *E. coli* by addition of the native riboswitch inducer, guanidine. As we theorized, guanidine addition to an IIL^S lab strain of *E. coli* substantially improved growth in the presence of IILs. The use of guanidine in scaled-up biochemical production by $sugE^+$ *E. coli* strains could provide an economical method for on-demand circumvention of IIL toxicity, avoiding the need for strain engineering.

Our investigation has revealed several transporters capable of conferring IIL tolerance to *E. coli*. Although the SMR transporters are classified as members of a protein family that is distinct from efflux pumps of the MFS superfamily, both types of pumps appear to function similarly in protecting bacteria from the toxic effects of certain ionic liquids. These discoveries may motivate future studies into establishing the best transporter (or transporters) to use in biofuel or commodity-chemical production strains of bacteria.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich.

Strains. A list of strains is given in Table S1 in the supplemental material, and the strains used for each figure are listed in Table S2 in the supplemental material.

***Bacillus* strain isolation.** Isolates from compost at the Jepson Prairie and Zamora green waste facilities (46) were screened for growth in 200 μl of LB with 294 mM (5%, wt/vol) [C_2C_1im][OAc] at 37°C. After 5 days of growth, wells showing growth were reserved for further analysis. Strains positive for growth were tested further on LB agar with 0.125 M, 0.25 M, and 0.5 M [C_2C_1im][OAc]. Only two isolates, JP5 (Jepson Prairie 5) and Z98 (Zamora 98), showed growth on the highest level of the IIL after 1 and 2 days, respectively. The 16S rRNA genes from each strain were amplified and sequenced using the U1 and U2 16S rRNA gene primers (47). Following 16S rRNA gene sequencing, these strains were renamed *Bacillus cereus* JP5 and *Bacillus licheniformis* Z98.

Growth analysis. All growth assays were performed in triplicate (unless otherwise noted) in EZ-rich medium (Teknova) with antibiotic (12.5 mg/ml chloramphenicol or 50 mg/ml kanamycin), IPTG (isopropyl-β-D-thiogalactopyranoside) inducer as appropriate, and/or IIL supplementation, as noted. Overnight cell cultures (biological triplicates) were diluted to an OD of 0.01 with medium, and 100 μl culture/well was grown in 96-well plates with shaking at 37°C in a plate reader. Absorbance reads at 600 nm were taken every 20 min. Absorbance data were background corrected by subtracting the minimum value for each sample. The maximum cell density (K) and maximum growth rate (μ_{max}) were modeled using a nonparametric local fit and graphed in R (v3.5.2). For visualization, growth curves were smoothed with locally estimated scatterplot smoothing (LOESS) and plotted with confidence interval bands.

Fosmid library construction and screening. Our fosmid library was prepared from genomic *B. cereus* JP5 DNA using the pCC1FOS fosmid vector (Epicentre Biotechnologies). Fosmid libraries were screened in *E. coli* EPI300 as previously described (17). Fosmids from positive *E. coli* clones were purified according to the Qiagen miniprep instructions, and chromosome regions inserts were identified by Sanger sequencing from the fosmid backbone into the isolated regions and then mapped to the appropriate genome.

Cloning and genome editing. A list of the vectors used is given in Table S3 in the supplemental material. Genes of interest were PCR amplified, cloned into BglBrick plasmids (48) using native or inducible plasmid-borne promoters as noted, and transformed into *E. coli*. *B. thuringiensis* mutations were made by two-step allelic replacement (25). For the first step, homology arms surrounding a gene targeted for knockout were cloned into the pBKJ236 integration vector and transformed into *E. coli* DH10b. The integration vectors were conjugated into *B. thuringiensis*, facilitated by the mating helper strain *E. coli* HB101(pRK600). The second step, including electroporation of pBKJ233, was performed as described previously. *B. thuringiensis* complementation vectors were prepared by cloning the designated genes into pHT304 (49). Complementation vectors were prepared from *dam dcm* mutant *E. coli* ER2925 (NEB) and electroporated into *B. thuringiensis* as described above. *E. coli* genome knockouts were prepared by amplifying Δ*sugE* from the Keio collection strain JW4144 (50) and transforming the PCR product into *E. coli* strains of interest carrying pKD46 (51). Unmarked deletions were prepared with pCP20 (50).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00069-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We are grateful to Jessica Bergmann and Bree Person for probing Jessica Bergmann's rainforest metagenome library for IIL tolerance phenotypes. We acknowledge Didier Lereclus (Institut National de la Recherche Agronomique, France) for providing pHT304 and Heather Jensen and Elizabeth Libby for technical advice. We thank Andrew Knappenberger for insightful discussions about the manuscript and ideas regarding regulatory mechanisms resulting from the mutations in the guanidine II riboswitch.

This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org), supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 with Lawrence Berkeley National Laboratory and contract DE-AC52-07NA27344 with Lawrence Livermore National Laboratory.

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