



BceAB-Type Antibiotic Resistance Transporters Appear To Act by Target Protection of Cell Wall Synthesis

 Carolin M. Kobras,^a Hannah Piepenbreier,^b Jennifer Emenegger,^c Andre Sim,^b  Georg Fritz,^{b*}  Susanne Gebhard^a

^aDepartment of Biology & Biochemistry, Milner Centre for Evolution, University of Bath, Bath, United Kingdom

^bLOEWE Center for Synthetic Microbiology and Department of Physics, Philipps-Universität Marburg, Marburg, Germany

^cDepartment Biologie I, Ludwig-Maximilians-Universität Munich, Munich, Germany

ABSTRACT Resistance against cell wall-active antimicrobial peptides in bacteria is often mediated by transporters. In low-GC-content Gram-positive bacteria, a common type of such transporters is BceAB-like systems, which frequently provide high-level resistance against peptide antibiotics that target intermediates of the lipid II cycle of cell wall synthesis. How a transporter can offer protection from drugs that are active on the cell surface, however, has presented researchers with a conundrum. Multiple theories have been discussed, ranging from removal of the peptides from the membrane and internalization of the drug for degradation to removal of the cellular target rather than the drug itself. To resolve this much-debated question, we here investigated the mode of action of the transporter BceAB of *Bacillus subtilis*. We show that it does not inactivate or import its substrate antibiotic bacitracin. Moreover, we present evidence that the critical factor driving transport activity is not the drug itself but instead the concentration of drug-target complexes in the cell. Our results, together with previously reported findings, lead us to propose that BceAB-type transporters act by transiently freeing lipid II cycle intermediates from the inhibitory grip of antimicrobial peptides and thus provide resistance through target protection of cell wall synthesis. Target protection has so far only been reported for resistance against antibiotics with intracellular targets, such as the ribosome. However, this mechanism offers a plausible explanation for the use of transporters as resistance determinants against cell wall-active antibiotics in Gram-positive bacteria where cell wall synthesis lacks the additional protection of an outer membrane.

KEYWORDS ABC transport, antimicrobial peptide, lipid II cycle, *Bacillus subtilis*

The bacterial cell wall and its biosynthetic pathway, the lipid II cycle, are important targets for antibiotics, especially in Gram-positive bacteria that lack the protective layer of the outer membrane. Cell wall-targeting drugs include antimicrobial peptides (AMPs), which bind to cycle intermediates and prevent biosynthetic enzymes from carrying out the next reaction (1). It is hardly surprising that bacteria have developed a plethora of strategies to protect themselves against such an antibiotic attack. Among the many known resistance mechanisms, a common strategy is the production of ATP-binding cassette (ABC) transporters that presumably remove AMPs from their site of action (2, 3). A major group of these is the BceAB-type transporters, which are found in many environmental and pathogenic species of the phylum *Firmicutes* (4). The eponymous and, to date, best-characterized system is BceAB of *Bacillus subtilis* (5). BceAB-type transporters comprise one permease (BceB) and two ATPases (BceA) (6). The permeases consist of 10 transmembrane helices and a large extracellular domain that is thought to contain the ligand-binding region of the transporter (7, 8). Transporter production is regulated via a two-component regulatory system (TCS) consisting of a histidine kinase (BceS) and a response regulator (BceR) (5, 7). A striking feature of

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Address correspondence to Susanne Gebhard, s.gebhard@bath.ac.uk.

* Present address: Georg Fritz, School of Molecular Sciences, The University of Western Australia, Perth, Western Australia, Australia.

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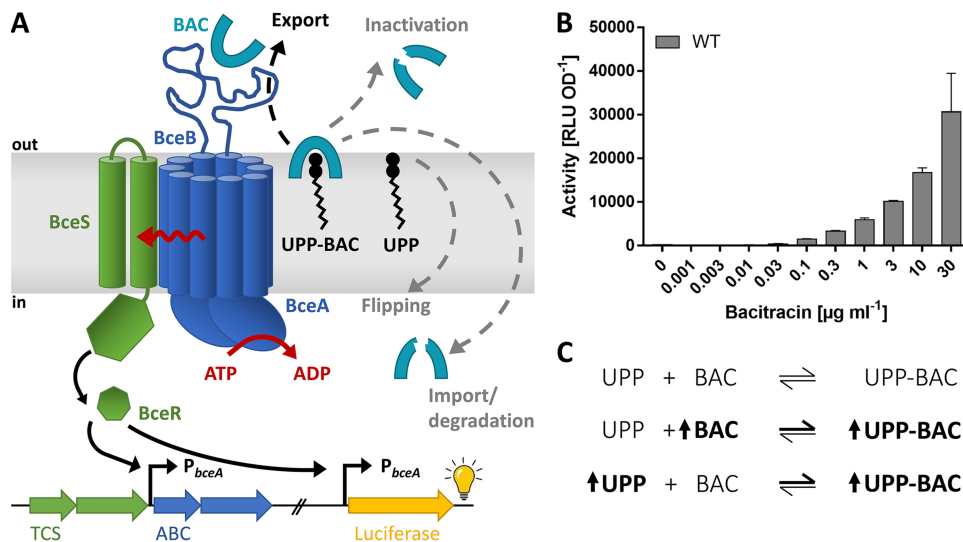


FIG 1 Antibiotic resistance and flux-sensing by BceAB. (A) Schematic of the BceAB-BceRS resistance system. The transporter BceAB confers resistance against bacitracin (BAC), which acts by binding its cellular target UPP. The different debated mechanisms for resistance by BceAB are indicated by dashed arrows (see text for details). Flux sensing communicates the transport activity of BceAB to the kinase BceS (red wave arrow), causing activation of BceR, which induces transcription from the target promoter P_{bceA} . This results in increased production of BceAB and, therefore, adjusted levels of resistance. As signaling is directly proportional to BceAB activity, we can use the target promoter P_{bceA} fused to a luciferase reporter to monitor transport activity. TCS, genes encoding the two-component regulatory system BceRS; ABC, genes encoding the resistance transporter BceAB. (B) Using luciferase activity as a proxy, BceAB activity of wild-type *B. subtilis* W168 carrying the P_{bceA} -*lux* reporter fusion (SGB73) was determined following 25- to 35-min challenge of exponentially growing cells with subinhibitory concentrations of bacitracin. All data are depicted as means \pm standard deviations of at least three biological replicates. (C) Binding reaction between free bacitracin and its cellular target UPP. The change in concentration of UPP-bacitracin complexes (UPP-BAC) through manipulation of either bacitracin or UPP concentrations is indicated by bold font and upward-facing arrows.

these systems is that signaling is triggered by the activity of the transporter itself (9). Due to this flux-sensing strategy, signaling is directly proportional to transport activity, and the transporter effectively autoregulates its own production (Fig. 1A).

BceAB confers resistance against the AMPs bacitracin, mersacidin, actagardine, and plectasin, of which bacitracin binds the lipid II cycle intermediate undecaprenyl pyrophosphate (UPP), while the others bind lipid II itself (5, 8). Considering the location of the AMPs' targets on the extracellular side of the cytoplasmic membrane, it is not immediately obvious how a membrane-embedded transporter can provide effective protection from these drugs. The mode of action of BceAB-type transporters has therefore been the subject of much debate (Fig. 1A). When first described, the *B. subtilis* system was named Bce for bacitracin efflux (5), although no evidence for the direction of transport was available. The assumption of export was based on the suggested self-protection mechanism of the unrelated transporter BcrAB in the bacitracin producer *Bacillus licheniformis* strain ATCC 10716 (10, 11). BcrAB was thought to work as a "hydrophobic vacuum cleaner" to remove the antibiotic from the membrane, akin to the human multidrug resistance transporter P-glycoprotein (12, 13). Later, BceAB was speculated to instead import bacitracin into the cytoplasm for subsequent degradation, again without direct experimental evidence (7). More recently, the transporter was proposed to act as a UPP flippase (14). In this scenario, BceAB would confer resistance by transporting UPP across the membrane to the cytoplasmic face, thereby removing the cellular target for bacitracin rather than transporting bacitracin itself. In the presence of bacitracin, BceAB was hypothesized to be inhibited by UPP-bacitracin complexes (UPP-BAC), which, in turn, should activate signaling through the BceRS two-component system to adjust BceAB levels in the cell (14). This model offered a neat explanation of the available data on bacitracin resistance but could not explain how the same transporter can confer resistance against AMPs that target lipid II instead of UPP.

Since then, we have shown that BceB is able to bind bacitracin *in vitro* (6). Without excluding the possibility of BceAB interacting with the UPP-BAC complex, this finding suggested that BceAB-like transporters directly interacted with the AMP and that the AMP is at least part of the physiological substrate. Moreover, the computational model used to establish the flux-sensing mechanism for signaling within the Bce system was based on the recognition of UPP-BAC complexes by the transporter and removal of bacitracin from the complex (9). Although the model did not specify a particular direction of transport, such a mechanism was most in line with the initial hydrophobic vacuum cleaner hypothesis (5). Resistance in this scenario is conferred by BceAB recognizing target AMP complexes in the membrane, removing the antibiotic, and releasing it into the extracellular milieu. This frees the target from the inhibitory action of the antibiotic and allows the next step of cell wall synthesis to proceed.

Considering the relevance of BceAB-like systems among *Firmicutes* bacteria, we here set out to address the controversial question on their mode of action and how a transporter can provide effective protection against cell surface-active antibiotics. Using a peptide release assay, we exclude that BceAB acts by import or inactivation of bacitracin. Based on the discovery that signaling within the Bce system is directly proportional to transport activity, we established a promoter-reporter assay as a proxy for transport activity. Our results show that the critical variable in determining transport activity of BceAB is bacitracin in complex with its cellular target UPP, rather than bacitracin or the lipid carrier alone. Taking together the findings of this study and the literature, we conclude that BceAB-type transporters appear to transiently free their cellular target from the inhibitory grip of the AMP and provide resistance via target protection of cell wall synthesis.

RESULTS AND DISCUSSION

BceAB does not import or inactivate bacitracin. To investigate the resistance mechanism of BceAB-type transporters, we first focused on the direction of transport by BceAB. To this end, we applied a modified version of the peptide release assay established by Otto and colleagues (15). This is based on the quantification of the AMP concentration that remains in the culture supernatant after incubating cell suspensions of bacteria carrying or lacking the transporter in an AMP-containing buffer. Presence of an importer should lead to a decrease in the AMP concentration remaining in the buffer, while an increase in AMP concentration compared to transporter-negative cells would be indicative of a mechanism where the drug is expelled from the bacterial cell envelope (15, 16). To quantify the remaining bacitracin, we chose a bioassay-based method, similar to the technique reported by Okuda and colleagues (17) (see Materials and Methods for details). This would allow us to determine the amount of biologically active peptide remaining to provide additional information on whether the action of BceAB may somehow inactivate the antibiotic.

Earlier models for BceAB action considered bacitracin import, potentially followed by intracellular degradation (7). Alternative conceivable mechanisms of resistance could be inactivation of the extracellular AMP, e.g., through shedding of phospholipids, which could be catalyzed by BceAB, akin to a mechanism reported for daptomycin resistance in *Staphylococcus aureus* (18). However, our bioassay methodology did not show any significant reduction in bacitracin activity by BceAB-containing cells (i.e., wild-type cells that had been preinduced with low concentrations of bacitracin to ensure *bceAB* expression), arguing against such mechanisms (Fig. 2). We observed a slight reduction in active bacitracin compared to the starting concentration of 5 $\mu\text{g ml}^{-1}$, but this applied to all samples, including the buffer control. Therefore, it was likely due to the known oxidative deamination of bacitracin A to bacitracin F, which lowers the antimicrobial activity (19) during incubation and sample processing. Our data thus indicate that BceAB neither imports bacitracin into the cell nor inactivates or degrades bacitracin in the extracellular space.

When we compared BceAB-positive and -negative cells, we could not detect significant differences in supernatant concentrations of the drug (Fig. 2). This was not in line

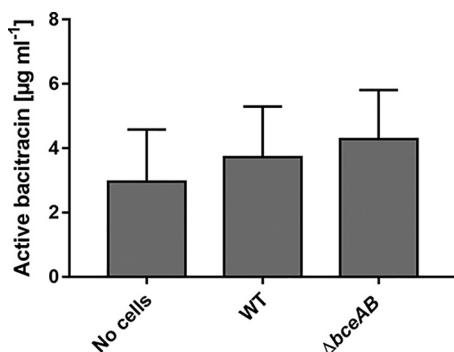


FIG 2 Bacitracin is neither imported nor inactivated by BceAB. Cell suspensions of OD₆₀₀ of 10 of *B. subtilis* W168 (WT) and an isogenic ΔbceAB mutant (TMB035), as well as a buffer control (no cells), were incubated with 5 $\mu\text{g ml}^{-1}$ bacitracin for 30 min. The biologically active bacitracin remaining in the supernatant after incubation was quantified using a bioassay. Data are shown as means \pm standard deviations of at least three biological replicates. One-way analysis of variance (ANOVA) did not show significant differences between samples.

with our hypothesis that BceAB should expel bacitracin from the membrane into the extracellular milieu. In a recent study on the BceAB-type transporter NsrFP from *Streptococcus agalactiae* COH1, which used a similar peptide release assay, the residual AMP concentration in the culture supernatant was significantly higher in an *nsrFP*⁺ strain than strains with no or inactive NsrFP, in agreement with a hydrophobic vacuum cleaner mechanism as proposed for BceAB (20). It is therefore tempting to speculate that BceAB works by a similar mechanism, considering that both proteins are closely related and members of the same type of transporters, even though our bioassay data are not conclusive on the direction of transport. The main difference between the previous work and our study was that the NsrFP experiments were done using the lantibiotic nisin as substrate, and earlier similar studies had also used lantibiotic substrates (15, 16, 21). As lantibiotics bind to lipid II while bacitracin binds UPP, it is to be expected that the kinetics of peptide binding and the proposed release via the transporter will differ between BceAB and NsrFP. In the case of BceAB, we believe that the peptide release assay may not have been sensitive enough to detect small differences in the amount of bacitracin attached to the cells.

Nevertheless, based on the homology between BceAB and NsrFP, it is plausible that both employ the same functional mechanism (20). Further support for the expulsion of AMPs from the membrane may be provided by the LanFEG-type transporters, which use such a strategy to confer self-immunity in AMP-producing bacteria. Well-known examples of this group include the transporters NisFEG of *Lactococcus lactis* and SpaFEG of *B. subtilis* (2). Several studies have shown that these transporters effectively mediate resistance against AMPs without degrading or inactivating the drugs but by releasing them into the culture supernatant (15–17, 21). Although LanFEG transporters share no close evolutionary relationship with BceAB-type systems (2), the fact that they impart resistance against the same range of antibiotics lends weight to the hypothesis that both use a similar principle of protection.

BceAB-type systems belong to the type VII ABC transporter superfamily, of which the *Escherichia coli* macrolide resistance transporter MacB is the paradigm example (22). MacB was recently shown to act according to a molecular bellows mechanism and expel its substrate from the periplasm across the outer membrane via the TolC exit duct by undergoing extensive conformational changes in its periplasmic domain (23). This mode of “transport,” which does not involve physical movement of a substrate across a membrane but instead uses intracellular ATP hydrolysis to perform mechanical work in the periplasm, was termed “mechanotransmission” (23). BceAB shares the critical features of MacB that are required for the mechanotransmission mechanism (22). In this case, the work carried out by the transporter would be to shift the equilibrium of the bacitracin-binding reaction from the membrane more toward the extracellular envi-

ronment. For such a hydrophobic vacuum cleaner mechanism to work, the transporter will need to distinguish between the membrane-bound and the free form of the AMP. Interestingly, bacitracin undergoes an extensive conformational change upon binding its cellular target, from a free state with no clear hydrophobic moment to an amphipathic, closed, dome-shaped conformation when bound to UPP (24). While we have shown previously that BceAB was able to bind bacitracin *in vitro*, these experiments were carried out with detergent-solubilized protein that may have contained copurified membrane lipids (6). We therefore cannot draw any direct conclusions on whether it interacted with the free drug or with any bacitracin-UPP complexes (UPP-BAC) that may have been present in the experiment. Therefore, we next aimed to identify the physiological substrate of BceAB *in vivo*.

Exploiting the flux-sensing mechanism as a suitable strategy to monitor BceAB activity. To study the function of BceAB *in vivo*, we first required a strategy to quantify transport activity in living cells. We previously showed that signaling within the Bce system is directly proportional to BceAB transport activity (9). As the signaling cascade ultimately leads to activation of the promoter controlling *bceAB* expression (P_{bceA}), the activity of a P_{bceA} -*luxABCDE* reporter fusion can therefore be taken as a proxy for BceAB activity (Fig. 1A). Using this approach, we monitored BceAB activity in the wild-type strain carrying the reporter fusion (SGB73) under several subinhibitory bacitracin concentrations. In agreement with earlier data (9), the threshold concentration to elicit detectable BceAB activity was $0.03 \mu\text{g ml}^{-1}$ bacitracin, and the activity gradually increased until maximum levels were reached at $30 \mu\text{g ml}^{-1}$ (Fig. 1B). As it was previously shown that higher bacitracin concentrations did not cause a further increase in activity (7, 9, 25), we deemed this concentration a suitable endpoint.

Accumulation of UPP specifically increases BceAB activity at low bacitracin concentrations. While the preliminary experiment in Fig. 1B showed that transport activity increased with higher bacitracin concentrations, it did not allow us to distinguish between free bacitracin and UPP-BAC as substrates. This is because the concentration of UPP-BAC will change proportionally with the concentration of bacitracin added to the culture (Fig. 1C). To distinguish whether the critical variable determining BceAB transport activity was bacitracin itself or the UPP-BAC complex, we required a strategy to change the concentration of UPP-BAC while keeping the concentration of bacitracin constant. Considering the reaction equilibrium between bacitracin and UPP-BAC (Fig. 1C), this should be possible by adjusting the cellular levels of UPP, as increased amounts of UPP result in higher concentrations of UPP-BAC without altering the bacitracin concentration.

To find a suitable genetic approach to change the UPP levels in the cell, we turned to mathematical modeling. Based on the computational description of the lipid II cycle (26), we recently developed a mathematical model that describes the protective effect of the bacitracin resistance determinants BceAB and BcrC of *B. subtilis* on the progression of the lipid II cycle (27). This model can predict changes to the pool levels of lipid II cycle intermediates under different conditions and has been verified by comparing predictions of growth inhibition by antibiotics targeting different steps of the cycle to corresponding experimental data (26, 27). The model suggests that reducing the rate of UPP dephosphorylation increases the level of UPP displayed on the extracellular face of the membrane. In *B. subtilis*, the dephosphorylation reaction of UPP to UP is catalyzed by two phosphatases, BcrC and UppP (28–30). BcrC plays a more prominent role during exponential growth, and *bcrC* deletion should thus have a bigger effect on reducing the rate of dephosphorylation. In a $\Delta bcrC$ scenario, the model predicted the UPP pool to increase more than 8-fold over the wild-type levels (Fig. 3A and B).

To exploit this finding, we deleted *bcrC* in our reporter strain ($\Delta bcrC$). When we retested this strain for BceAB activity, we observed a striking 10-fold reduction in the threshold concentration required to trigger detectable transport activity ($0.003 \mu\text{g ml}^{-1}$) (Fig. 3C). Likewise, maximum BceAB activity was observed at $0.3 \mu\text{g ml}^{-1}$ bacitracin (Fig. 3C, turquoise), 100-fold less than required to reach a similar activity in the wild type (Fig. 3C, dark blue). Fitting of a dose-response curve to the normalized

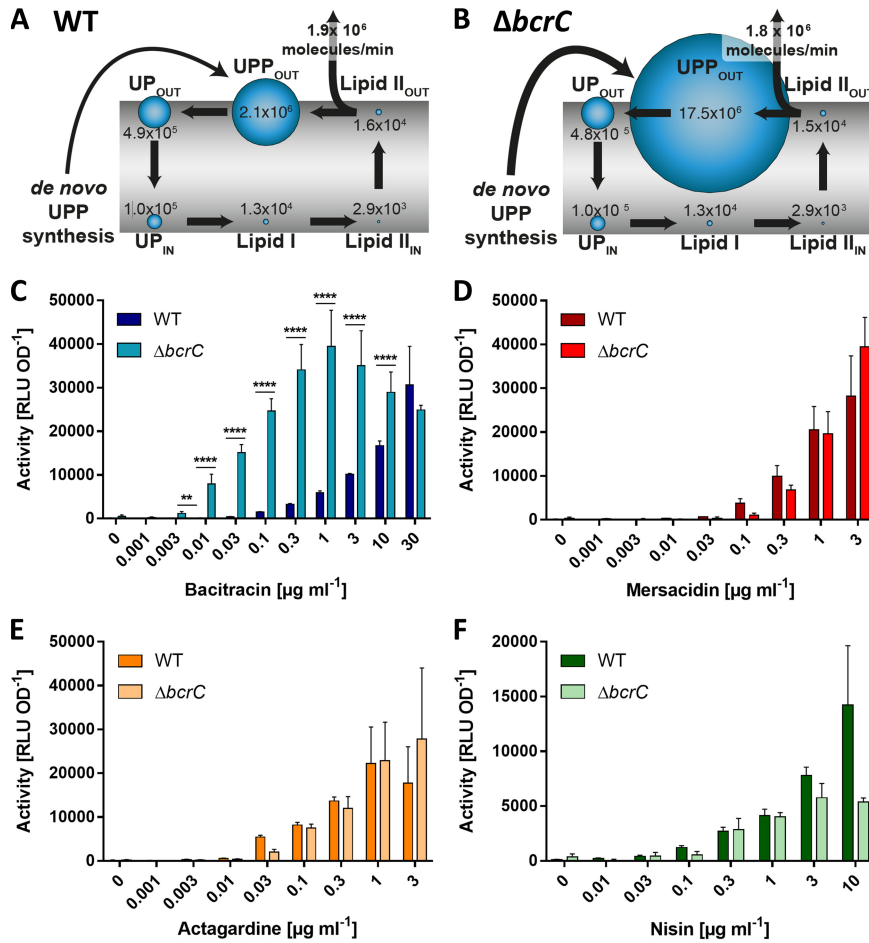


FIG 3 Accumulation of UPP increases transport activity at low bacitracin concentrations but does not affect activity on lipid II-binding AMPs. (A, B) Pool levels of lipid II cycle intermediates, as predicted by mathematical modeling, are indicated by the relative size of blue bubbles and numbers of molecules per cell for each intermediate are given. The rate of peptidoglycan (PG) synthesis is shown in molecules of precursor incorporated per minute. The thickness of the arrow for *de novo* UPP synthesis reflects the previously described homeostatic increase in lipid carrier synthesis upon *bcrC* deletion (27). Wild type (A) and *bcrC* deletion mutant (B). (C to F) Effect of UPP accumulation on transport activity *in vivo*. As a proxy for transport, luminescence activities of P_{bceA} -*lux* (C, D) or P_{psdA} -*lux* (F) reporter strains were determined 25 to 35 min following the challenge of exponentially growing cells with varying concentrations of AMPs as indicated. Each panel shows the results for one AMP given below the *x* axis. Dark bars show results in the wild-type background (SGB73 or SGB74); lighter bars show results in the isogenic $\Delta bcrC$ background (SGB649 or SGB681). Data are shown as means \pm standard deviations of at least three biological replicates. The increased activity seen in the $\Delta bcrC$ background compared to wild type was tested for statistical significance using two-sided *t* tests with *post hoc* Bonferroni-Dunn correction for multiple comparisons (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $0.01 < P < 0.05$).

data for both strains (see Methods and Materials for details) showed that, indeed, the half-maximal effective concentration of bacitracin (EC_{50}), i.e., the concentration where BceAB activity was half its maximum, was shifted from $5.5 \mu\text{g ml}^{-1}$ in the wild type to $0.05 \mu\text{g ml}^{-1}$ in the $\Delta bcrC$ strain (Fig. S1A in the supplemental material). Importantly, the overall shape of the curve was not altered between strains, showing that the differences were solely due to changes in substrate concentration upon UPP accumulation, not any mechanistic changes in the transporter itself that may have been caused by *bcrC* deletion. Moreover, we had previously shown that a $\Delta bcrC$ strain complemented with an ectopic copy of *bcrC* showed signaling behavior that was indistinguishable from the wild type, ruling out any polar effects of the deletion (25). To explore if UPP alone could serve as the physiological substrate of BceAB, the activity was also compared in the absence of bacitracin. There was no detectable BceAB activity in either of the tested strains, which suggested that accumulation of UPP alone was not

sufficient to trigger transport by BceAB. These findings were a first indication that the critical variable that determines BceAB activity is the concentration of UPP-BAC complexes, rather than bacitracin or UPP alone.

In the wild type, induction of P_{bceA} of course not only drives reporter gene expression but also increases the amount of BceAB present in the cell. To exclude that the observed sensitivity shift upon UPP accumulation was not due to changes in *bceAB* expression, we uncoupled BceAB production from its native regulation. This was achieved by deleting the native copy of *bceAB* in the reporter strain and introducing an ectopic copy under xylose-inducible control (P_{xyIA} -*bceAB*; strain SGB218). The same was done in the $\Delta bcrC$ reporter, resulting in strain SGB677. Comparing BceAB activity in these two strains again showed a marked decrease (30-fold) in the threshold bacitracin concentration required to trigger detectable activity upon accumulation of UPP (Fig. S1B). This shows that the observed shift in sensitivity of BceAB could not be explained by indirect regulatory effects on *bceAB* expression.

Accumulation of UPP may have caused wider alterations in the cell membrane and/or affected BceAB activity in a nonspecific manner rather than the intended change in the concentration of UPP-BAC complexes. Therefore, we next tested if *bcrC* deletion also altered BceAB activity in response to AMPs that do not interfere with UPP. To this end, we measured BceAB activity in response to mersacidin and deoxyactagardine B, two other AMPs that are known substrates for BceAB (8). As both of these peptides target lipid II but not UPP, the complex formation between these AMPs and their respective cellular targets should be unaffected by changes in the UPP level. Indeed, our theoretical model predicted that the lipid II pool on the extracellular face of the membrane would remain almost unchanged in a $\Delta bcrC$ scenario compared to the wild type (Fig. 3A and B). This is because the total amount of lipid carrier is homeostatically increased in a *bcrC* deletion strain to ensure a close-to-optimal rate of peptidoglycan synthesis (27). The model therefore confirms that decreasing the UPP dephosphorylation rate in the $\Delta bcrC$ strain specifically causes accumulation of UPP but does not affect other cycle intermediates.

As with bacitracin, a gradual increase of BceAB activity was observed with increasing amounts of mersacidin or deoxyactagardine B in both the wild-type and $\Delta bcrC$ strains (Fig. 3D and E). However, we did not observe any significant differences in threshold substrate concentrations or overall BceAB activity between the two strains. As an additional control, we tested the activity of a second BceAB-type transporter in *B. subtilis*, PsdAB, which confers resistance against nisin, another lipid II-binding AMP (8). PsdAB activity was determined using the same luminescence-based assay principle as for BceAB but with P_{psdA} activity as a proxy for transport activity. As before, activity increased with nisin concentration in both the wild type and $\Delta bcrC$ mutant, but again, no significant differences between strains were observed (Fig. 3F). These findings show that *bcrC* deletion and concomitant accumulation of UPP did not have a general effect on BceAB or PsdAB function. Instead, BceAB activity appeared to specifically depend on the concentration of UPP-BAC in the membrane. This is consistent with the proposed hypothesis for a hydrophobic vacuum cleaner mechanism of transport, suggesting that the physiological substrate of BceAB is indeed the antibiotic in complex with its cellular target.

In the case of its lipid II-binding target drugs, e.g., mersacidin, we postulate that the substrate recognized by BceAB should be, by analogy, the lipid II-mersacidin complex. The concentration of these complexes should be unaltered in the *bcrC* deletion strains, as discussed above, but should instead respond to changes in lipid II concentrations. However, any attempts at changing the concentration of lipid II in the cell, e.g., by deletion of penicillin-binding proteins (PBPs), have so far been unsuccessful. This is presumably due to redundancy between multiple PBPs and is also consistent with our mathematical model, which showed that the large UPP pool in the cycle can act as an effective buffering reservoir to compensate for interference with the much smaller lipid II pool (26, 27).

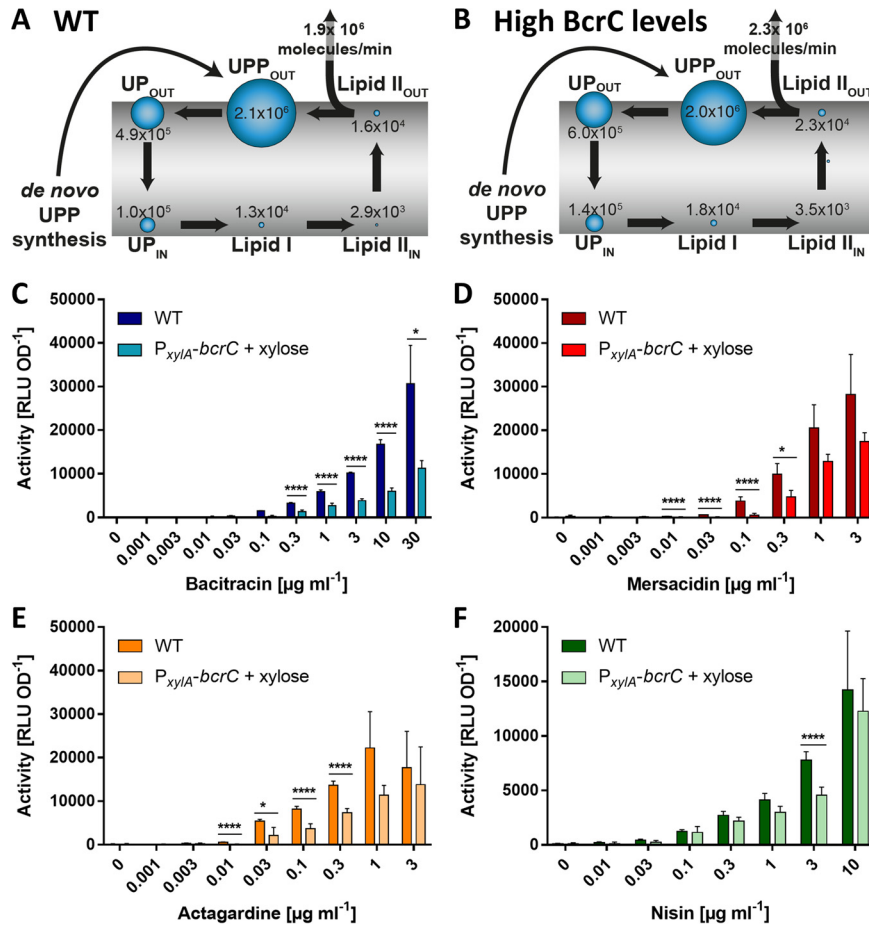


FIG 4 Attempted depletion of UPP has a global negative effect on transport. (A, B) Pool levels of lipid II cycle intermediates, as predicted by mathematical modeling, are indicated by the relative size of blue bubbles, and numbers of molecules per cell for each intermediate are given. The rate of peptidoglycan (PG) synthesis is shown in molecules of precursor incorporated per minute. Wild type (A) and BcrC overproduction strain (B). (C to F) Effect of UPP depletion on transport activity *in vivo*. As a proxy for transport, luminescence activities of P_{bceA}-lux (C to E) or P_{psdA}-lux (F) reporter strains were determined 25 to 35 min following the challenge of exponentially growing cells with varying concentrations of AMPs as indicated. Each panel shows the results for one AMP given below the x axis. Dark bars show results in the wild-type background (SGB73 or SGB74); lighter bars show a strain overproducing BcrC (SGB758 or SGB974). Data are shown as means ± standard deviations of at least three biological replicates. Tests for statistical significance of differences in activity in the overproduction versus wild-type backgrounds were done by two-sided *t* test with *post hoc* Bonferroni-Dunn correction for multiple comparisons (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $0.01 < P < 0.05$).

Attempted depletion of UPP affects transport activity on a global level. To further explore the effect of altered UPP levels on BceAB activity, we next sought to decrease the pool of UPP displayed on the outer face of the membrane and hence the number of UPP-BAC complexes formed. The mathematical model predicted that an increased rate of UPP dephosphorylation, e.g., by overproducing BcrC, may lead to such a decreased UPP pool (Fig. 4A and B), although differences to the wild type are less pronounced than with the *bcrC* deletion. To realize this experimentally, we overproduced BcrC by placing an additional copy of *bcrC* under the control of the xylose-inducible promoter P_{xyIA} (strain SGB758). Testing the BceAB activities in the strain with reduced UPP levels led to overall lower activity upon addition of bacitracin, and even at the maximal concentration tested, the activity was less than 50% of the wild-type activity (Fig. 4C). The threshold concentration required to trigger detectable activity was only marginally increased. Fitting the normalized activities with a dose-response curve produced identical results for both strains (Fig. S1C). This suggests that BcrC overproduction led to an overall decrease in BceAB activity but had no effect on the

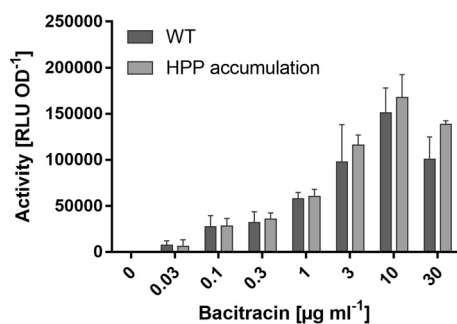


FIG 5 Accumulation of HPP does not inhibit BceAB activity. Transport activities, using luciferase activity of the P_{bceA} -*lux* reporter as a proxy, were determined for the WT (SGB927, dark gray) and an HPP accumulation strain ($\Delta ytpB \Delta menA amyE::P_{spac(hy)}-menA$, SGB929, light gray) grown in MCSE minimal medium 25 to 35 min following exposure to varying bacitracin concentrations. Data are shown as means \pm standard deviations of at least three biological replicates. Two-sided *t* tests with *post hoc* Bonferroni-Dunn correction for multiple comparisons did not show any significant difference between the wild-type and the HPP accumulation strains.

transporter's sensitivity to the substrate. Also consistent with a more global effect of BcrC overproduction on cell physiology was the observation that BceAB activity was similarly reduced when mersacidin and deoxyactagardine B were tested (Fig. 4D and E). Likewise, the activity of PsdAB using nisin as a substrate was also reduced (Fig. 4F). It therefore appears that overproduction of BcrC did not have the desired effect of solely reducing the UPP pool in the cell but instead led to wider-ranging changes that affected either multiple stages of the lipid II cycle, explaining similar effects on bacitracin and lipid II-binding AMPs, or impeded the mechanical functions of the membrane-embedded transporters to reduce their overall activity. Importantly, cell viability and growth rates following antibiotic exposure with any of the compounds tested here were not affected by BcrC overproduction (Fig. S2), ruling out that the lower transport activity was somehow caused by cell death.

Without further knowledge of the precise cellular effects of BcrC overproduction, it is difficult to interpret these results. However, while they do not further support our hypothesis that BceAB recognizes its substrate AMP as a complex with the cellular target, they also do not disprove it.

Accumulation of C_{35} isoprenoid heptaprenyl diphosphate does not inhibit BceAB activity. In addition to the theories on bacitracin import, export or inactivation by BceAB-type transporters, a drastically different mechanism has been proposed where BceAB could protect the cell from bacitracin by flipping UPP from the outer leaflet of the membrane to the inner face, thereby shielding it from the AMP (14). This hypothesis was based on the observation that accumulation of the C_{35} isoprenoid heptaprenyl diphosphate (HPP) in the membrane sensitizes the cell to bacitracin. HPP was hypothesized to act as a competitive inhibitor of BceAB and to reduce its transport activity. To explore this hypothesis further, we next tested the effect of HPP accumulation on BceAB activity, using the luciferase-based assay described above. Accumulation of HPP can be created by manipulations of the isoprenoid biosynthesis pathway, specifically via deletion of *ytpB*, which encodes a tetraprenyl-beta-curcumene synthase (53), and simultaneous limitation of the activity of MenA, a key enzyme in the menaquinone pathway. The *menA* gene is essential, but a reduction in enzymatic activity can be achieved by growth in tryptophan-limited conditions (14). Therefore, to determine whether BceAB activity is inhibited by HPP accumulation, we tested BceAB activity in a $\Delta ytpB \Delta menA$ deletion strain that carried an ectopic IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible copy of *menA* (SGB929, based on HB13438) and was grown in a tryptophan-limited defined medium without the addition of IPTG.

Interestingly, the threshold bacitracin concentration required to trigger transport, as well as the activity at peak stimulation, were indistinguishable between the two strains, showing that HPP accumulation did not affect BceAB activity (Fig. 5). To confirm that

our strategy had led to the desired HPP accumulation, we tested the bacitracin sensitivity of both strains. The MIC decreased from $173 \pm 12 \mu\text{g ml}^{-1}$ in the wild type to $120 \mu\text{g ml}^{-1}$ in the mutant strain, in line with the previously reported increased susceptibility upon HPP accumulation (14). Based on these results, we concluded that the increased bacitracin sensitivity was not due to direct inhibition of BceAB activity by HPP. Instead, our interpretation is that BceAB likely cannot distinguish between UPP-BAC and HPP-BAC. Bacitracin was shown to tightly interact with the pyrophosphate group and only the first isoprenoid unit of its substrate, based on its cocrystal structure with the C_{10} isoprenoid geranyl pyrophosphate (24). It is therefore expected that HPP will also serve as a bacitracin target in the cell, and its accumulation will lead to the simultaneous presence of both UPP-BAC and HPP-BAC complexes. In the context of our findings above that UPP-BAC—and by analogy, also HPP-BAC—is the likely physiological substrate of BceAB, it is plausible that either complex will drive BceAB activity. Hence, accumulation of HPP did not affect the net transport activity. However, HPP cannot substitute for UPP in the lipid II cycle. Any activity of BceAB invested in the removal of bacitracin from HPP is therefore futile with respect to resistance, which can explain the increased bacitracin sensitivity observed upon HPP accumulation. Taking together, our findings, along with the previous detailed study of the effects of HPP accumulation on bacitracin resistance (14), a model where BceAB removes bacitracin from its cellular targets appears more in line with the available experimental evidence than a UPP-flipping mechanism. Furthermore, as mentioned, BceAB also confers resistance against lipid II-binding AMPs, namely, mersacidin, actagardine, and the fungal defensin plectasin (8, 31). For these compounds, it is difficult to envisage a flipping mechanism as an effective strategy to shield the target from AMP access because import of lipid II runs counter the process of cell wall biosynthesis, where peptidoglycan precursors are required on the surface of the membrane.

In this study, we set out to address the much-debated question on the mode of action of BceAB-type resistance systems and how a transporter may be used to protect the cell from antibiotics that have targets on the cell surface. The balance of evidence presented here and in the literature appears to be in clear favor of BceAB acting as a hydrophobic vacuum cleaner, which is in line with the mechanotransmission mechanism proposed for type VII superfamily ABC systems (22, 23). In this model, BceAB specifically recognizes its substrate AMPs in complex with their respective cellular target, here experimentally tested for UPP-BAC. ATP hydrolysis in the cytoplasm then provides the required energy to break the interaction between bacitracin and UPP on the cell surface. This is not a novel concept in a transporter because the human cholesterol transporter ABCG5/8 employs a similar mechanism to remove cholesterol from the cytoplasmic membrane of hepatocytes, using the energy from ATP hydrolysis to break the interactions between cholesterol and membrane phospholipids (32). In the case of BceAB, a shift in equilibrium from target-bound AMP to free AMP can be achieved if the transporter has a low affinity to the free antibiotic and therefore releases it as soon as it is removed from the target. Given the substantial conformational change of bacitracin and other peptides between the free and target-bound forms (1, 33–35), this seems entirely plausible. In support of this idea, we have only been able to show binding of bacitracin by the entire detergent-solubilized transporter, where UPP may have been copurified (6), but never with its isolated extracellular domain that provides substrate specificity in BceAB-like systems (36) and therefore is thought to contain the ligand-binding site (our unpublished data).

How then does a simple shift in equilibrium confer the high level of resistance that is the hallmark of BceAB-like transporters? For one, there is ample evidence that the LanFEG-type transporters of AMP-producing bacteria work by exactly such a mechanism to provide effective protection from the self-produced AMP (15–17, 21). Moreover, a similar principle, albeit not on the cell surface, is seen in resistance against tetracyclines, a group of antibiotics that target the bacterial ribosome. Here, ribosomal protection proteins like Tet(O) and Tet(M) were shown to actively release tetracycline from the ribosome in a GTP-driven manner (37, 38). This mechanism effectively

increases the dissociation rate of tetracycline and secures continued protein synthesis (39). Interestingly, another resistance system that protects ribosomal function from antibiotic attack is a group of proteins referred to as antibiotic resistance ATP-binding cassette-F (ARE ABC-F). While originally annotated as transporters, these proteins lack any transmembrane segments and instead act by modulating the binding affinity between antibiotics and the ribosome, thus effectively dislodging the drugs (40, 41). This mode of resistance has been collectively termed “target protection” and generally involves the direct release of a cellular target from the inhibitory action of the antibiotic (41, 42). Target protection has been reported for the ribosome and DNA replication (43–45), but to our knowledge, no example has been described to date for the protection of cell wall synthesis. We now propose that BceAB-type transporters act by target protection of the lipid II cycle. By physically freeing UPP from the grip of bacitracin (or analogously, freeing lipid II from lantibiotics), BceAB ensures that the affected enzyme (UPP-phosphatase or peptidoglycan [PG] transglycosylases, respectively) can catalyze the following step of cell wall synthesis, enabling the cycle to continue at least for one more round before the antibiotic can rebind its target. Importantly, to our knowledge, this mode of action is in agreement with all experimental data currently available on BceAB-like systems. Recognition of target-AMP complexes, rather than the free peptides, offers an explanation for the seemingly random substrate specificity of BceAB-like systems (8, 46), where the specificity determinant likely only becomes apparent in the antibiotic-target complex. It also explains the observations on reduced resistance upon overproduction of HPP in the cell, where some of BceAB’s transport activity is likely invested in the futile removal of bacitracin from HPP rather than UPP (14). It is consistent with the data reported here and previously (9, 27) that the factor determining the transport activity of BceAB is the concentration of UPP-BAC complexes in the cell. Target protection of cell wall synthesis also offers a plausible explanation for the use of transporters in resistance against cell wall-active antibiotics in Gram-positive bacteria. Whereas the outer membrane of Gram-negative microorganisms creates a discrete compartment, and transporters can be used to change a compound’s concentration in this space, Gram-positive bacteria lack an equivalent structure. It will be interesting to explore if other transport systems in these bacteria operate by a similar mechanism to protect the cell wall synthesis machinery from antibiotic attack.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are given in Table 1. *E. coli* and *B. subtilis* strains were routinely grown at 37°C with agitation (180 rpm) in lysogeny broth (LB) medium. Solid media contained 1.5% (wt/vol) agar. Selective media contained ampicillin (100 $\mu\text{g ml}^{-1}$), chloramphenicol (5 $\mu\text{g ml}^{-1}$), kanamycin (10 $\mu\text{g ml}^{-1}$), spectinomycin (100 $\mu\text{g ml}^{-1}$), tetracycline (10 $\mu\text{g ml}^{-1}$), or erythromycin (1 $\mu\text{g ml}^{-1}$) with lincomycin (25 $\mu\text{g ml}^{-1}$, macrolide-lincosamide-streptogramin B [mls]). For full induction of the promoter P_{xyjA} , xylose was added to a final concentration of 0.2% (wt/vol). Bacterial growth was routinely monitored as optical density at a wavelength of 600 nm (OD_{600}) measured spectrophotometrically in cuvettes of 1 cm light path length.

Strain construction and molecular cloning. All plasmids used in this study are listed in Table 1; primer sequences are given in Table 2. *B. subtilis* transformations were performed using a modified version of the Paris protocol (47). Overnight cultures of recipient strains were grown in 500 μl Paris medium (6.1 mM K_2HPO_4 , 4.4 mM KH_2PO_4 , 0.4 mM trisodium citrate, 1% [wt/vol] glucose, 20 mM potassium L-glutamate, 0.1% [wt/vol] Casamino Acids, 3 mM MgSO_4 , 25 $\mu\text{g ml}^{-1}$ tryptophan, and 8 μM ferric ammonium citrate) at 37°C with aeration (180 rpm). Day cultures (500 μl) were inoculated 1:50 in fresh, prewarmed Paris medium and grown for 3 h (37°C, 180 rpm). To each culture, 50 μl of isolated genomic DNA (gDNA) of the donor strain, or 0.5 to 1 μg of isolated plasmid DNA, was added. Transformation cultures were grown for 5 more hours and plated on selective media. For mls or chloramphenicol resistance, cultures were preinduced for 1 h at 1:40 of the final concentration of the respective antibiotic. Donor strain gDNA was isolated by mixing an overnight culture of the donor 1:1 with sodium citrate (SC) buffer (150 mM NaCl, 10 mM sodium citrate, pH 7.0) and harvesting the cells by centrifugation (5 min, $17,800 \times g$). The pellet was resuspended in SC buffer and incubated with lysozyme at 37°C for 15 min. The solution was mixed 1:1 with 5 M NaCl and passed through a 0.45- μm syringe-driven filter. Plasmid DNA was isolated from *E. coli* using conventional mini-prep kits.

To create a construct for inducible expression of *bcrC*, the gene was PCR amplified from *B. subtilis* W168 using primers TM2731 and TM2732, which incorporated prefix and suffix, respectively, of the modified “Freiburg standard” of BioBrick cloning described previously (48). The resulting fragment was

TABLE 1 Plasmids and bacterial strains used in this study

Plasmid or strain	Genotype or description ^a	Source or reference
Plasmid		
pAH328	Vector for transcriptional promoter fusions to <i>luxABCDE</i> ; integrates in <i>B. subtilis</i> <i>sacA</i> ; Amp ^r , Cm ^r	52
pBS2E	Empty vector; integrates in <i>B. subtilis</i> <i>lacA</i> ; Amp ^r , mls ^r	48
pBS3Elux	Vector for transcriptional promoter fusions to <i>luxABCDE</i> ; integrates in <i>B. subtilis</i> <i>sacA</i> ; Amp ^r , Mls ^r	50
pSB1A3	Empty BioBrick standard cloning vector for <i>E. coli</i> ; Amp ^r	Registry of Standard Biological Parts
pSDLux101	pAH328 harboring a transcriptional P _{bceA} - <i>luxABCDE</i> fusion	49
pSDLux102	pAH328 harboring a transcriptional P _{psdA} - <i>luxABCDE</i> fusion	This study
pJNESB101	pSB1A3 harboring <i>B. subtilis</i> <i>bcrC</i> in BioBrick format	This study
pJNE2E01	pBS2E harboring a transcriptional P _{xyIA} - <i>bcrC</i> fusion, assembled according to the BioBrick RFC25 cloning standard	This study
pNT2E01	pBS2E harboring P _{xyIA} - <i>bceAB</i> , assembled according to the BioBrick RFC10 cloning standard	9
pMG3Elux1	pBS3Elux harboring a transcriptional P _{bceA} - <i>luxABCDE</i> fusion	This study
<i>B. subtilis</i> strain		
W168	Wild type, <i>trpC2</i>	Laboratory stock
TMB035 ($\Delta bceAB$)	W168 <i>bceAB</i> ::kan; Kan ^r	7
TMB297 ($\Delta bcrC$)	W168 <i>bcrC</i> ::tet; Tet ^r	7
TMB713 ($\Delta bceAB \Delta bcrC$)	W168 <i>bceAB</i> ::kan <i>bcrC</i> ::tet; Kan ^r , Tet ^r	25
HB13350	W168 <i>ytpB</i> ::spec; Spec ^r	14
HB13438	W168 <i>menA</i> ::kan <i>amyE</i> ::P _{spac(hy)} - <i>menA</i> ; Kan ^r , Cm ^r	14
SGB73	W168 <i>sacA</i> ::pSDLux101; Cm ^r	9
SGB74	W168 <i>sacA</i> ::pSDLux102; Cm ^r	This study
SGB218	W168 <i>bceAB</i> ::kan <i>sacA</i> ::pSDLux101 <i>lacA</i> ::pNT2E01; Kan ^r , Cm ^r , Mls ^r	9
SGB243	W168 <i>lacA</i> ::pJNE2E01; Mls ^r	This study
SGB649	W168 <i>bcrC</i> ::tet <i>sacA</i> ::pSDLux101; Tet ^r , Cm ^r	This study
SGB677	W168 <i>bceAB</i> ::kan <i>bcrC</i> ::tet <i>sacA</i> ::pSDLux101 <i>lacA</i> ::pNT2E01; Kan ^r , Tet ^r , Cm ^r , Mls ^r	This study
SGB681	W168 <i>bcrC</i> ::tet <i>sacA</i> ::pSDLux102; Tet ^r , Cm ^r	This study
SGB758	W168 <i>sacA</i> ::pSDLux101 <i>lacA</i> ::pJNE2E01; Cm ^r , Mls ^r	This study
SGB873	W168 <i>menA</i> ::kan <i>amyE</i> ::P _{spac(hy)} - <i>menA</i> <i>ytpB</i> ::spec; Kan ^r , Cm ^r , Spec ^r	This study
SGB927	W168 <i>sacA</i> ::pMG3Elux1; Mls ^r	This study
SGB929	W168 <i>menA</i> ::kan <i>amyE</i> ::P _{spac(hy)} - <i>menA</i> <i>ytpB</i> ::spec <i>sacA</i> ::pMG3Elux1; Kan ^r , Cm ^r , Spec ^r , Mls ^r	This study
SGB974	W168 <i>sacA</i> ::pSDLux102 <i>lacA</i> ::pJNE2E01; Cm ^r , Mls ^r	This study

^aAmp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Mls^r, macrolide, lincosamide, and streptogramin B resistance; Tet^r, tetracycline resistance; Spec^r, spectinomycin resistance.

cloned into pSB1A3 via the EcoRI and PstI restriction sites (pJNESB101). The *bcrC* gene was then reexcised using XbaI and PstI. Assembly with an EcoRI/SpeI fragment of the BioBrick carrying the xylose-inducible promoter P_{xyIA} (48) into EcoRI/PstI digested pBS2E resulted in the inducible P_{xyIA}-*bcrC* construct pJNE2E01. A transcriptional P_{psdA}-*lux* reporter construct (pSDLux102) was created by PCR amplification of the promoter region of *psdAB* of *B. subtilis* using primers TM0599 and TM2242 and ligation with pAH328 via the EcoRI and NotI restriction sites. The existing P_{bceA}-*luxABCDE* reporter (pSDLux101) (49) was reconstructed in vector pBS3Elux (50), which contains an mls resistance marker instead of chloramphenicol. This was achieved by PCR amplification of the promoter fragment with primers SG843 and SG883 and cloning via EcoRI and PstI sites, resulting in plasmid pMG3Elux1.

Determination of the MIC. The susceptibility of *B. subtilis* strains to bacitracin was determined using the MIC determined by broth microdilutions. For this, 2-fold serial dilutions of Zn²⁺-bacitracin were prepared in 2 ml of Mueller-Hinton medium and inoculated 1:500 from overnight cultures grown in the same medium. For higher resolution, in some instances, defined concentrations of Zn²⁺- bacitracin were added directly to each culture. Cultures were incubated overnight (37°C, 180 rpm) and examined for growth after 24 h. The MIC was determined as the lowest concentration at which no visible growth was detected. All experiments were performed in at least biological triplicates, and mean values and standard deviations were calculated to report the data.

Bacitracin uptake assays. Bacitracin uptake was assayed with slight modification to previously described protocols (15, 16). Overnight cultures were diluted 1:500 in 100 ml LB supplemented with 1% (wt/vol) fructose. To induce BceAB production in the wild type, 1 μg ml⁻¹ bacitracin was added at the time of inoculation. The cultures were incubated for 3.5 to 4.75 h at 37°C (200 rpm) until they reached an OD₆₀₀ of 1.0 to 2.0. Cells were harvested by centrifugation (4,000 × g, 10 min, room temperature) and washed twice with 50 mM potassium phosphate (pH 7 to 7.5) and 100 mM NaCl. Cell density was adjusted to an OD₆₀₀ of 10 in assay buffer (50 mM potassium phosphate [pH 7 to 7.5], 100 mM NaCl, 1% [wt/vol] fructose, and 50 μM zinc sulfate). Aliquots of 2.4 ml of the cell suspension were incubated for 10 min at 37°C (200 rpm). Bacitracin was added to a final concentration of 5 μg ml⁻¹, followed by incubation for 30 min at 37°C (200 rpm). As a control, one sample containing no cells received identical treatment. Cells were removed by centrifugation (4,000 × g, 10 min, room temperature), and the supernatants were filtered (0.45 μm). The supernatants were stored for no longer than 5 days at -20°C and were concentrated 5-fold using an Eppendorf Concentrator 5301 speed vacuum at room temperature.

TABLE 2 Primers used in this study

Primer	Description/use ^a	Sequence (5'–3') ^b	Source or reference
SG0148	<i>lacA</i> insertion, fwd	GCATACCGTTGCCGTCATC	This study
SG0149	<i>lacA</i> insertion, rev	GAACATCATGCACTCCACAC	This study
SG0506	<i>amyE</i> insertion, fwd	GTAAGCGTTAACAAAATTCTC	This study
SG0507	<i>amyE</i> insertion, rev	TTATATTGTGCAACACTTCACA	This study
SG0528	<i>sacA</i> insertion up, fwd	CTGATTGGCATGGCGATTGC	48
SG0529	<i>sacA</i> insertion up, rev	ACAGCTCCAGATCCTCTACG	48
SG0530	<i>sacA</i> insertion down, fwd	GTCGCTACCATTACCAGTTG	48
SG0531	<i>sacA</i> insertion down, rev	TCCAAACATTCCGGTGTATC	48
SG0630	<i>ytpB</i> up, fwd	TCATGTGGACCTGGAAAGCA	14
SG0633	<i>ytpB</i> do, rev	TGATCGTCCACCGCATTACA	14
SG0637	<i>menA</i> up, fwd	CCGTACACAAGGATAGGAGA	14
SG0640	<i>menA</i> do, rev	GAAGGCGAAAGCATCTGACA	14
SG0842	P _{bceAr} fwd EcoRI	CAC GAATTC GAACATGTCATAAGCGTGTGACG	This study
SG0883	P _{bceAr} rev PstI	CGG ACTGCA GTATCGATGCCCTTCAGCACTTCC	This study
TM0599	P _{psdAr} fwd EcoRI	AGT CGAATTC CACCCTCGTGAATGTGACAGC	This study
TM2242	P _{psdAr} rev NotI	AATT GCGGCCG CGATAGGTTCTGTTTGTGCAACACG	This study
TM2731	<i>bcrC</i> Biobrick, fwd	GAT CGAATTC GCGGCCGCT TCTAGAA AAGGAGGT GCGCCG CTGAACTACGAAATTTTAAAGCAATC	This study
TM2732	<i>bcrC</i> Biobrick, rev	GAT CTACTAGT ATTAA ACCGGT GAAATTTTATCGGTTGGTTTTTTC	This study

^afwd, forward; rev, reverse.

^bSequences in bold highlight restriction sites used for cloning.

To quantify the bacitracin remaining in the culture supernatants, the sensitivity of the strain TMB713 was exploited in a bioassay adapted from the method established by Okuda et al. (51). To this end, an overnight culture of TMB713, grown in LB with selective antibiotics, was diluted 1:30 into 3 ml melted (60°C) LB soft agar (0.75% [wt/vol]) and poured evenly onto a dried LB agar plate, allowed to solidify 10 min at room temperature, and then dried for 10 min. Plugs 6 mm in diameter were removed from the plate, leaving stable holes in the agar. In volumes of 50 μ l, bacitracin standards (5 to 50 μ g ml⁻¹) and concentrated supernatants were applied into the holes, and plates were immediately incubated upright at 37°C. After 24 to 26 h, the diameter of the growth inhibition zone was measured. Clearing zones measured from bacitracin standards were used to create a standard curve. Bacitracin concentrations in supernatants were extrapolated using the standard curve and worked back to the original sample from the known 5-fold concentration factor during sample preparation.

Computational model and simulations. Model predictions for the data in Fig. 3 and 4 were performed with a previously established model for the lipid II cycle and its interaction with the bacitracin stress response network in *B. subtilis* (26, 27). Briefly, the model uses deterministic differential equations to describe the time-dependent concentrations of the different lipid II cycle intermediates, as well as the bacitracin stress response modules BcrC and BceAB. A detailed description of the model assumptions and equations for the bacitracin resistance network in *B. subtilis* wild-type and the $\Delta bcrC$ mutant has been laid out before (27). In the model for the $\Delta bcrC$ mutant, a homeostatic upshift in *de novo* synthesis of UPP leads to maintenance of PG synthesis to ensure *bcrC* deletion is not lethal (27). This additional increase in the carrier pool exacerbates the accumulation of UPP even further. To illustrate the model behavior for a *bcrC* overexpression strain (Fig. 4B), we assumed that this strain features a 1.5-fold stronger UPP phosphatase activity than wild-type cells, based on the higher activity of the P_{xyIA} promoter driving *bcrC* expression in this strain (9) relative to the native P_{bcrC} promoter (25). All numerical simulations of the differential equations were performed with custom scripts developed in MATLAB software (MathWorks, Inc.).

Luciferase reporter assay. For reporter gene assays, 10 ml of LB or modified chemically defined medium (MCSE, as described in reference 16) were inoculated 1:1,000 from overnight cultures of each strain to be tested. Day cultures were grown at 37°C with agitation (180 rpm) to an OD₆₀₀ of around 0.5 to ensure exponential growth. Cultures were then diluted into fresh growth medium to an OD₆₀₀ of 0.01 and distributed into 96-well microplates (Corning; black, clear, flat bottom), with 100 μ l culture volume per well. Wells around the plate edge were filled with water to reduce evaporation. Luciferase activity of strains was determined in a Tecan Spark microplate reader controlled by the SparkControl software (Tecan Trading AG, Switzerland). Cells were grown in the microplate reader for 5 h with continuous shaking incubation (37°C; 180 rpm; orbital motion; amplitude, 3 mm). After 1 h of incubation, cells were challenged with varying concentrations of antibiotic. The OD₆₀₀ and luminescence (relative luminescence units [RLU]) were measured every 5 min (integration time, 1,000 ms). Luminescence output was normalized to cell density by dividing each data point by its corresponding blank-corrected OD₆₀₀ value (RLU OD⁻¹). For dose-response curves, RLU OD⁻¹ values were determined from the average of three data points taken at steady state (25, 30, and 35 min). Experiments were carried out at least in biological triplicates. To determine the dose-response behavior of strains for bacitracin, luminescence values were normalized, with 0% defined as the lowest and 100% as the highest measured RLU OD⁻¹ value for each strain. Data were then fitted with variable-slope dose-response curves in GraphPad Prism7, using the logarithms of bacitracin concentrations as *x* and normalized luminescence as *y* values and applying default settings. Statistical comparison of the resulting EC₅₀ values was performed using the in-built

comparison tool for nonlinear regression fits of GraphPad Prism 7, based on an extra sum-of-squares F test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES

- Breukink E, de Kruijff B. 2006. Lipid II as a target for antibiotics. *Nat Rev Drug Discov* 5:321–332. <https://doi.org/10.1038/nrd2004>.
- Gebhard S. 2012. ABC transporters of antimicrobial peptides in *Firmicutes* bacteria - phylogeny, function and regulation. *Mol Microbiol* 86:1295–1317. <https://doi.org/10.1111/mmi.12078>.
- Revilla-Guarinos A, Gebhard S, Mascher T, Zúñiga M. 2014. Defence against antimicrobial peptides: different strategies in *Firmicutes*. *Environ Microbiol* 16:1225–1237. <https://doi.org/10.1111/1462-2920.12400>.
- Dintner S, Staron A, Berchtold E, Petri T, Mascher T, Gebhard S. 2011. Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in *Firmicutes* bacteria. *J Bacteriol* 193:3851–3862. <https://doi.org/10.1128/JB.05175-11>.
- Ohki R, Giyanto, Tateno K, Masuyama W, Moriya S, Kobayashi K, Ogasawara N. 2003. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. *Mol Microbiol* 49:1135–1144. <https://doi.org/10.1046/j.1365-2958.2003.03653.x>.
- Dintner S, Heermann R, Fang C, Jung K, Gebhard S. 2014. A sensory complex consisting of an ATP-binding cassette transporter and a two-component regulatory system controls bacitracin resistance in *Bacillus subtilis*. *J Biol Chem* 289:27899–27910. <https://doi.org/10.1074/jbc.M114.596221>.
- Rietkötter E, Hoyer D, Mascher T. 2008. Bacitracin sensing in *Bacillus subtilis*. *Mol Microbiol* 68:768–785. <https://doi.org/10.1111/j.1365-2958.2008.06194.x>.
- Staron A, Finkeisen DE, Mascher T. 2011. Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*. *Antimicrob Agents Chemother* 55:515–525. <https://doi.org/10.1128/AAC.00352-10>.
- Fritz G, Dintner S, Treichel NS, Radeck J, Gerland U, Mascher T, Gebhard S. 2015. A new way of sensing: need-based activation of antibiotic resistance by a flux-sensing mechanism. *mBio* 6:e00975. <https://doi.org/10.1128/mBio.00975-15>.
- Podlesek Z, Comino A, Herzog-Velikonja B, Zgur-Bertok D, Komel R, Grabnar M. 1995. *Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance. *Mol Microbiol* 16:969–976. <https://doi.org/10.1111/j.1365-2958.1995.tb02322.x>.
- Podlesek Z, Comino A, Herzog-Velikonja B, Grabnar M. 2000. The role of the bacitracin ABC transporter in bacitracin resistance and collateral detergent sensitivity. *FEMS Microbiol Lett* 188:103–106. <https://doi.org/10.1111/j.1574-6968.2000.tb09176.x>.
- Higgins CF, Gottesman MM. 1992. Is the multidrug transporter a flippase? *Trends Biochem Sci* 17:18–21. [https://doi.org/10.1016/0968-0004\(92\)90419-a](https://doi.org/10.1016/0968-0004(92)90419-a).
- Gottesman MM, Pastan I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 62:385–427. <https://doi.org/10.1146/annurev.bi.62.070193.002125>.
- Kingston AW, Zhao H, Cook GM, Helmann JD. 2014. Accumulation of heptaprenyl diphosphate sensitizes *Bacillus subtilis* to bacitracin: implications for the mechanism of resistance mediated by the BceAB transporter. *Mol Microbiol* 93:37–49. <https://doi.org/10.1111/mmi.12637>.
- Otto M, Peschel A, Götz F. 1998. Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of *Staphylococcus epidermidis* TÜ3298. *FEMS Microbiol Lett* 166:203–211. <https://doi.org/10.1111/j.1574-6968.1998.tb13891.x>.
- Stein T, Heinzmann S, Solovieva I, Entian KD. 2003. Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*. *J Biol Chem* 278:89–94. <https://doi.org/10.1074/jbc.M207237200>.
- Okuda K, Aso Y, Nakayama J, Sonomoto K. 2008. Cooperative transport between NukFEG and NukH in immunity against the lantibiotic nukacin ISK-1 produced by *Staphylococcus warneri* ISK-1. *J Bacteriol* 190:356–362. <https://doi.org/10.1128/JB.01300-07>.
- Pader V, Hakim S, Painter KL, Wigneshweraraj S, Clarke TB, Edwards AM. 2016. *Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. *Nat Microbiol* 2:16194. <https://doi.org/10.1038/nmicrobiol.2016.194>.
- Storm DR, Strominger JL. 1973. Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions. *J Biol Chem* 248:3940–3945.
- Reiners J, Lagedroste M, Ehlen K, Leusch S, Zschke-Kriesche J, Smits S. 2017. The N-terminal region of nisin is important for the BceAB-type ABC transporter NsrFP from *Streptococcus agalactiae* COH1. *Front Microbiol* 8:1643. <https://doi.org/10.3389/fmicb.2017.01643>.
- Stein T, Heinzmann S, Dusterhus S, Borchert S, Entian KD. 2005. Expression and functional analysis of the subtilin immunity genes *spalFEG* in the subtilin-sensitive host *Bacillus subtilis* MO1099. *J Bacteriol* 187:822–828. <https://doi.org/10.1128/JB.187.3.822-828.2005>.
- Greene NP, Kaplan E, Crow A, Koronakis V. 2018. Antibiotic resistance mediated by the MacB ABC transporter family: a structural and functional perspective. *Front Microbiol* 9:950. <https://doi.org/10.3389/fmicb.2018.00950>.
- Crow A, Greene NP, Kaplan E, Koronakis V. 2017. Structure and mechanotransmission mechanism of the MacB ABC transporter superfamily. *Proc Natl Acad Sci U S A* 114:12572–12577. <https://doi.org/10.1073/pnas.1712153114>.
- Economou NJ, Cocklin S, Loll PJ. 2013. High-resolution crystal structure reveals molecular details of target recognition by bacitracin. *Proc Natl Acad Sci U S A* 110:14207–14212. <https://doi.org/10.1073/pnas.1308268110>.
- Radeck J, Gebhard S, Orchard PS, Kirchner M, Bauer S, Mascher T, Fritz G.

2016. Anatomy of the bacitracin resistance network in *Bacillus subtilis*. *Mol Microbiol* 100:607–620. <https://doi.org/10.1111/mmi.13336>.
26. Piepenbreier H, Diehl A, Fritz G. 2019. Minimal exposure of lipid II cycle intermediates triggers cell wall antibiotic resistance. *Nat Commun* 10:2733. <https://doi.org/10.1038/s41467-019-10673-4>.
 27. Piepenbreier H, Sim A, Kobras CM, Radeck J, Mascher T, Gebhard S, Fritz G. 2019. From modules to networks: A systems-level analysis of the bacitracin stress response in *Bacillus subtilis*. *bioRxiv* <https://doi.org/10.1101/827469>.
 28. Cao M, Helmann JD. 2002. Regulation of the *Bacillus subtilis* bcrC bacitracin resistance gene by two extracytoplasmic function σ factors. *J Bacteriol* 184:6123–6129. <https://doi.org/10.1128/jb.184.22.6123-6129.2002>.
 29. Zhao H, Sun Y, Peters JM, Gross CA, Garner EC, Helmann JD. 2016. Depletion of undecaprenyl pyrophosphate phosphatases disrupts cell envelope biogenesis in *Bacillus subtilis*. *J Bacteriol* 198:2925–2935. <https://doi.org/10.1128/JB.00507-16>.
 30. Radeck J, Lautenschlager N, Mascher T. 2017. The essential UPP phosphatase pair BcrC and UppP connects cell wall homeostasis during growth and sporulation with cell envelope stress response in *Bacillus subtilis*. *Front Microbiol* 8:2403. <https://doi.org/10.3389/fmicb.2017.02403>.
 31. Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK, Mygind PH, Raventos DS, Neve S, Ravn B, Bonvin AM, De Maria L, Andersen AS, Gammelgaard LK, Sahl HG, Kristensen HH. 2010. Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. *Science* 328:1168–1172. <https://doi.org/10.1126/science.1185723>.
 32. Small DM. 2003. Role of ABC transporters in secretion of cholesterol from liver into bile. *Proc Natl Acad Sci U S A* 100:4–6. <https://doi.org/10.1073/pnas.0237205100>.
 33. Hsu ST, Breukink E, Bierbaum G, Sahl HG, de Kruijff B, Kaptein R, van Nuland NA, Bonvin AM. 2003. NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles. Conformational changes are a key to antimicrobial activity. *J Biol Chem* 278:13110–13117. <https://doi.org/10.1074/jbc.M211144200>.
 34. Fujinami D, Mahin AA, Elsayed KM, Islam MR, Nagao J, Roy U, Momin S, Zendo T, Kohda D, Sonomoto K. 2018. The lantibiotic nukacin ISK-1 exists in an equilibrium between active and inactive lipid-II binding states. *Commun Biol* 1:150. <https://doi.org/10.1038/s42003-018-0150-3>.
 35. Medeiros-Silva J, Jekhmene S, Paioni AL, Gawarecka K, Baldus M, Swiezewska E, Breukink E, Weingarth M. 2018. High-resolution NMR studies of antibiotics in cellular membranes. *Nat Commun* 9:3963. <https://doi.org/10.1038/s41467-018-06314-x>.
 36. Hiron A, Falord M, Valle J, Debarbouille M, Msadek T. 2011. Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters. *Mol Microbiol* 81:602–622. <https://doi.org/10.1111/j.1365-2958.2011.07735.x>.
 37. Burdett V. 1996. Tet(M)-promoted release of tetracycline from ribosomes is GTP dependent. *J Bacteriol* 178:3246–3251. <https://doi.org/10.1128/jb.178.11.3246-3251.1996>.
 38. Trieber CA, Burkhardt N, Nierhaus KH, Taylor DE. 1998. Ribosomal protection from tetracycline mediated by Tet(O): Tet(O) interaction with ribosomes is GTP-dependent. *Biol Chem* 379:847–855. <https://doi.org/10.1515/bchm.1998.379.7.847>.
 39. Connell SR, Tracz DM, Nierhaus KH, Taylor DE. 2003. Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother* 47:3675–3681. <https://doi.org/10.1128/aac.47.12.3675-3681.2003>.
 40. Sharkey LKR, Edwards TA, O'Neill AJ. 2016. ABC-F proteins mediate antibiotic resistance through ribosomal protection. *mBio* 7:e01975. <https://doi.org/10.1128/mBio.01975-15>.
 41. Sharkey LKR, O'Neill AJ. 2018. Antibiotic resistance ABC-F proteins: bringing target protection into the limelight. *ACS Infect Dis* 4:239–246. <https://doi.org/10.1021/acscinfed.7b00251>.
 42. Wilson DN, Wilson DN. 2016. The ABC of ribosome-related antibiotic resistance. *mBio* 7:e00598-16. <https://doi.org/10.1128/mBio.00598-16>.
 43. Ruiz J. 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *J Antimicrob Chemother* 51:1109–1117. <https://doi.org/10.1093/jac/dkg222>.
 44. Tran JH, Jacoby GA, Hooper DC. 2005. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. *Antimicrob Agents Chemother* 49:3050–3052. <https://doi.org/10.1128/AAC.49.7.3050-3052.2005>.
 45. Correia S, Poeta P, Hebraud M, Capelo JL, Igrejas G. 2017. Mechanisms of quinolone action and resistance: where do we stand? *J Med Microbiol* 66:551–559. <https://doi.org/10.1099/jmm.0.000475>.
 46. Gebhard S, Mascher T. 2011. Antimicrobial peptide sensing and detoxification modules: unravelling the regulatory circuitry of *Staphylococcus aureus*. *Mol Microbiol* 81:581–587. <https://doi.org/10.1111/j.1365-2958.2011.07747.x>.
 47. Kunst F, Rapoport G. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J Bacteriol* 177:2403–2407. <https://doi.org/10.1128/jb.177.9.2403-2407.1995>.
 48. Radeck J, Kraft K, Bartels J, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebhard S, Mascher T. 2013. The *Bacillus* BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *J Biol Eng* 7:29. <https://doi.org/10.1186/1754-1611-7-29>.
 49. Kallenberg F, Dintner S, Schmitz R, Gebhard S. 2013. Identification of regions important for resistance and signalling within the antimicrobial peptide transporter BceAB of *Bacillus subtilis*. *J Bacteriol* 195:3287–3297. <https://doi.org/10.1128/JB.00419-13>.
 50. Popp PF, Dotzler M, Radeck J, Bartels J, Mascher T. 2017. The *Bacillus* BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. *Sci Rep* 7:15058. <https://doi.org/10.1038/s41598-017-15107-z>.
 51. Okuda K, Yanagihara S, Sugayama T, Zendo T, Nakayama J, Sonomoto K. 2010. Functional significance of the E loop, a novel motif conserved in the lantibiotic immunity ATP-binding cassette transport systems. *J Bacteriol* 192:2801–2808. <https://doi.org/10.1128/JB.00003-10>.
 52. Schmalisch M, Maiques E, Nikolov L, Camp AH, Chevreux B, Muffler A, Rodriguez S, Perkins J, Losick R. 2010. Small genes under sporulation control in the *Bacillus subtilis* genome. *J Bacteriol* 192:5402–5412. <https://doi.org/10.1128/JB.00534-10>.
 53. Sato T, Yoshida S, Hoshino H, Tanno M, Nakajima M, Hoshino T. 2011. Sesquiterpenes (C₁₅ terpenes) biosynthesized via the cyclization of a linear C₁₅ isoprenoid by a tetraprenyl-beta-curcumene synthase and a tetraprenyl-beta-curcumene cyclase: identification of a new terpene cyclase. *J Am Chem Soc* 133:9734–9737.