Identification of Regions Important for Resistance and Signalling within the Antimicrobial Peptide Transporter BceAB of Bacillus subtilis

Felix Kallenberg, Sebastian Dintner, Roland Schmitz and Susanne Gebhard

Published Ahead of Print 17 May 2013.

Updated information and services can be found at:
http://jb.asm.org/content/195/14/3287

These include:

REFERENCES
This article cites 30 articles, 8 of which can be accessed free at:
http://jb.asm.org/content/195/14/3287#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Identification of Regions Important for Resistance and Signalling within the Antimicrobial Peptide Transporter BceAB of *Bacillus subtilis*

Felix Kallenberg, Sebastian Dintner, Roland Schmitz, Susanne Gebhard

Ludwig-Maximilians-Universität München, Department Biology I, Microbiology, Martinsried, Germany

In the low-G+C-content Gram-positive bacteria, resistance to antimicrobial peptides is often mediated by so-called resistance modules. These consist of a two-component system and an ATP-binding cassette transporter and are characterized by an unusual mode of signal transduction where the transporter acts as a sensor of antimicrobial peptides, because the histidine kinase alone cannot detect the substrates directly. Thus, the transporters fulfill a dual function as sensors and detoxification systems to confer resistance, but the mechanistic details of these processes are unknown. The paradigm and best-understood example for this is the BceRS-BceAB module of *Bacillus subtilis*, which mediates resistance to bacitracin, mersacidin, and actagardine. Using a random mutagenesis approach, we here show that mutations that affect specific functions of the transporter BceAB are primarily found in the C-terminal region of the permease, BceB, particularly in the eighth transmembrane helix. Further, we show that while signaling and resistance are functionally interconnected, several mutations could be identified that strongly affected one activity of the transporter but had only minor effects on the other. Thus, a partial genetic separation of the two properties could be achieved by single amino acid replacements, providing first insights into the signaling mechanism of these unusual modules.

Many bacteria produce antibiotics to gain a competitive advantage over other microorganisms inhabiting the same ecological niche. Among the low-G+C-content Gram-positive bacteria, the *Firmicutes*, production of antimicrobial peptides is widely distributed. These compounds generally act by an inhibition of the lipid II cycle of cell wall synthesis (1, 2). For example, lantibiotics such as nisin or mersacidin bind to lipid II directly (1–3), whereas the non-ribosomally synthesized bacitracin prevents the dephosphorylation and recycling of the lipid carrier undecaprenyl-pyrophosphate (4). For self-protection, producer strains of antimicrobial peptides express so-called immunity proteins, summarily referred to as LanI, as well as ATP-binding cassette (ABC) transporters of the LanFEG or BcrAB type (5–8).

In order to compete successfully with such strains, nonproducing *Firmicutes* bacteria have also developed resistance mechanisms, the most efficient of which are again ABC transporters. However, these transporters differ in sequence and domain architecture from those of the producer strains and are collectively referred to as BceAB-type transporters (8, 9). They consist of an ATPase (BceA) and a permease (BceB) with 10 transmembrane helices and a characteristic, large extracellular domain of approximately 200 amino acids between helices VII and VIII. The eponymous and best-characterized example is BceAB of *Bacillus subtilis*, which confers resistance to bacitracin, mersacidin, and actagardine (10, 11). BceAB-type transporters are found in a conserved genomic arrangement with two-component systems (referred to as BceRS-like) whose histidine kinases possess two transmembrane helices but lack any extracellular sensory domains (9, 12, 13). Together, the transporter and the two-component system constitute peptide antibiotic resistance modules. Their most striking property is the unusual mode of signal transduction: the histidine kinases alone cannot detect the presence of antimicrobial peptides but instead rely on the transporters for stimulus perception. In the presence of a substrate peptide, the transporter somehow communicates with the sensor kinase, which leads to activation of the cognate response regulator and, subsequently, an induction of transporter gene expression. Importantly, ATP hydrolysis by the transporter and therefore active transport are required for the signaling process (10, 14). Experimental evidence from a number of homologous systems from *B. subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Lactobacillus casei* confirms this signaling pathway as a general characteristic of the Bce-type modules (10, 11, 14–17). Based on the coevolution of the proteins involved and supported by bacterial two-hybrid analyses of the VraFG transporter and GraRS two-component system of *S. aureus*, it is thought that communication within these modules involves direct protein-protein contacts between the transporter and histidine kinase (9, 15, 18). However, no information is available regarding the mechanism of signaling.

In this study, we performed a random mutagenesis of the transport permease BceB of *B. subtilis* with the aim to identify regions or residues within the transporter that are involved in signaling and/or resistance. A central question was whether amino acid replacements could be identified that affected only one but not the other function of BceB. In other words, are the two traits genetically separable in a dual-function transporter such as BceAB? We show that a partial separation of signaling and resistance could be obtained from single point mutations and further identify the C-terminal region of the transport permease as functionally important.
### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *B. subtilis* and *Escherichia coli* were routinely grown in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). During cloning of bacterial two-hybrid constructs, all media for *E. coli* were supplemented with 0.4% (wt/vol) glucose. Transformations of *B. subtilis* were carried out as described previously (19). All strains used in this study are listed in Table 1. Selective media contained kanamycin (10 µg ml⁻¹ for *B. subtilis*, 50 µg ml⁻¹ for *E. coli*), spectinomycin (100 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹), or ampicillin (100 µg ml⁻¹). Solid media contained 1.5% (wt/vol) agar.

**Plasmid construction.** All plasmids created during this study are listed in Table 1; all other strains are referred to as their wild-type counterparts, with the amino acid exchange given in parentheses.

To create a plasmid for xylose-inducible expression of *bceAB* in *B. subtilis* (pSG704), a 2.8-kb fragment containing the entire operon, including the ribosome binding site, was PCR amplified using primers 0011 and 1359 and cloned via the XbaI and KpnI sites into vector pKT25, resulting in a translational fusion of the CyaA T25 fragment to the N terminus of *bceAB* (pFK727). A similar construct expressing *bceA* to the C terminus of *BceS* (pAS1803) was created using primers 0011 and 1360 and cloned via the XbaI and KpnI sites into vector pKT25, resulting in a translational fusion of the *lucABCD* (luciferase) promoter to *bceB* (pAS1804). The resulting product was cloned as a BamHI fragment into vector pXT, placing the genes under the control of the vector’s promoter. A similar construct expressing *bceAB* to the C terminus of *BceS* (pAS1804) was also created using primers 0011 and 1360 and cloned via the XbaI and KpnI sites into vector pKT25, resulting in a translational fusion of the *lucABCD* (luciferase) promoter to *bceB* (pAS1805). The resulting product was cloned into the EcoRI and NotI sites of vector pSDlux101, creating plasmid pSDlux101.

To create a transcriptional fusion of the *bceA* promoter, *P<sub>bceA</sub>* to a promoterless luciferase operon (*luxABCD*), a 207-bp fragment encompassing the promoter region from 127 bp upstream to 80 bp downstream of the *bceA* start codon was PCR amplified with primers 0554 and 2241. The resulting product was cloned into the EcoRI and NotI sites of vector pAH328 (21), creating plasmid pSDlux101.

To create a plasmid for xylose-inducible expression of *bceA*, a 1.2-kb DNA fragment containing *bceS* was amplified via PCR using primers 1355 and 1356 and cloned into the EcoRI and NotI sites of vector pETDuet-1, creating plasmid pETDuet-1-bceS.

**Random chemical mutagenesis.** To generate random mutations in *bceB*, 10 µg of plasmid DNA of pER703 (10) was mixed with 10 vol of HA solution (1 M hydroxylamine hydrochloride, 100 mM NaCl, 50 mM sodium phosphate [pH 6], 2 mM EDTA [pH 8]) and incubated at 75°C for 15 min followed by purification with a HighYield PCR-clean up kit (Süd-Laborbedarf, Gauting, Germany). Mutagenized plasmid DNA was then linearized with Scal, and 2 µg of this was used to transform *B. subtilis* strain TMB301 with selection for resistance to kanamycin and spectinomycin. The resulting transformants were replica stamped with velvet pads onto indicator plates containing xylose (0.2% [wt/vol]), 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) (200 µg ml⁻¹), and Zn²⁺-bacitracin (Sigma; 0.5 µg ml⁻¹). All white colonies were again replica plated onto indicator plates and selective media (kanamycin, spectinomycin, chloramphenicol) to confirm the phenotype. Positive clones (i.e., those showing white colonies and resistance to all antibiotics) were then tested for threonine auxotrophy to confirm correct integration of the *lux* fragment into *thcB*. For this, each clone was inoculated into MNGE medium [100 mM glucose, 80 mM K₂HPO₄, 45 mM KH₂PO₄, 10 mM potassium glutamate, 4 mM sodium acetate, 3 mM MgSO₄, 50 µg ml⁻¹ tryptophan, and 40 µM Fe(III)-ammonium citrate], with and without kanamycin resistance; *Spcr* 23

### TABLE 1 Plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAC6</td>
<td>Vector for transcriptional promoter fusions to <em>lacZ</em>; integrates at <em>amyE</em>; <em>Cm</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>pAH328</td>
<td>Vector for transcriptional promoter fusions to <em>luxABCD</em> (luciferase); integrates in <em>sacA</em>; <em>Cm</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>pKT25</td>
<td>Translational fusion of <em>cyaA</em> T25 fragment to N terminus of insert polypeptide; <em>lac</em> promoter; <em>Kan</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>pUT18</td>
<td>Translational fusion of <em>cyaA</em> T18 fragment to C terminus of insert polypeptide; <em>lac</em> promoter; <em>Amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>pXT</td>
<td>Vector for xylose-inducible gene expression; integrates in <em>thcB</em>; <em>Sp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td>pAS1803</td>
<td>pUT18-<em>bceS</em></td>
<td>This study</td>
</tr>
<tr>
<td>pAS1804</td>
<td>pUT18-<em>bceA</em></td>
<td>This study</td>
</tr>
<tr>
<td>pAS2505</td>
<td>pKT25-<em>bceB</em></td>
<td>This study</td>
</tr>
<tr>
<td>pEF603</td>
<td>pAC6-<em>P&lt;sub&gt;bceA&lt;/sub&gt;</em>-<em>lacZ</em></td>
<td>10</td>
</tr>
<tr>
<td>pER703</td>
<td>pXT-<em>bceB</em></td>
<td>10</td>
</tr>
<tr>
<td>pFK727</td>
<td>pXT-<em>bceAB</em>-FLAG&lt;sub&gt;₆&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSDlux101</td>
<td>pAH328-*P&lt;sub&gt;bceA&lt;/sub&gt;-<em>luxABCD</em></td>
<td>This study</td>
</tr>
<tr>
<td>pSG704</td>
<td>pXT-<em>bceAB</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td><em>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F&lt;sup&gt;+&lt;/sup&gt;:Tn10 proAB lacY&lt;sup&gt;+&lt;/sup&gt; Δ</em>(lacZ)M15]*</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BTH101</td>
<td><em>F&lt;sup&gt;−&lt;/sup&gt; cya-99 araD139 galE15 galK16 rpsL1 hsdR2 mcrA1 mcrB1</em></td>
<td>Euromedex</td>
</tr>
<tr>
<td><em>B. subtilis</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W168</td>
<td>Wild type, <em>trpC2</em></td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SGB079</td>
<td>W168 <em>bceAB</em>:<em>Kan sacA</em>::<em>SDlux101</em></td>
<td>This study</td>
</tr>
<tr>
<td>SGB082</td>
<td>W168 <em>bceAB</em>:<em>Kan sacA</em>::<em>SDlux101</em> <em>thcC</em>::<em>SG704</em></td>
<td>This study</td>
</tr>
<tr>
<td>SGB170</td>
<td>W168 <em>bceAB</em>:*Kan thrC::<em>SG704</em></td>
<td>This study</td>
</tr>
<tr>
<td>SGB176</td>
<td>W168 <em>bceAB</em>:*Kan thrC::<em>FK727</em></td>
<td>This study</td>
</tr>
<tr>
<td>TMB035</td>
<td>W168 <em>bceAB</em>:<em>Kan</em></td>
<td>10</td>
</tr>
<tr>
<td>TMB301</td>
<td>W168 <em>bceB</em>: <em>Kan any</em>::<em>ER603</em></td>
<td>10</td>
</tr>
<tr>
<td>TMB378</td>
<td>W168 <em>bceB</em>: <em>Kan any</em>::<em>ER603</em> <em>thcC</em>::<em>ER703</em></td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only constructs with WT sequence inserts and derived strains are listed; mutated constructs and strains are named according to their amino acid exchanges throughout the article. *Amp*<sup>+</sup>, ampicillin resistance; *Cm*<sup>+</sup>, chloramphenicol resistance; *Kan*<sup>+</sup>, kanamycin resistance; *Sp*<sup>+</sup>, spectinomycin resistance.
addition of 50 µg mL⁻¹ threonine. From each clone that was unable to
grow in the absence of threonine, the introduced bceB gene was reamplified
by colony PCR using pXT-specific primers, and the resulting product was
sequenced to identify any introduced mutations.

### Site-directed mutagenesis.
For characterization of the mutations, each amino acid exchange identified by the random mutagenesis ap-
proach was reconstructed by site-directed mutagenesis of pSG704. Se-
lected amino acid exchanges were also introduced into pFK727 and
pAS2505. Primer design and mutagenesis were performed according to
the manufacturer’s instructions for the QuikChange II site-directed mu-
tagenesis kit (Agilent Technologies). Where this procedure was not suc-
cessful, mutations were introduced using the PCR-overlap extension
method (24), followed by cloning into the desired vector as described for
plasmid construction above. All plasmids were sequenced to confirm in-
troduction of the desired mutation.

### Reporter gene assays.
Luciferase activities of strains harboring pSDlux101 were assayed using a Synergy2 multimode microplate reader from
BioTek. The reader was controlled using the software Gen5. LB
medium was inoculated at 1:1,000 from overnight cultures of reporter
strains, and each strain was grown in a 100-µL volume in four wells of a
96-well plate. Cultures were incubated at 37°C with agitation (intensity,
200) were monitored every 5 min. OD 600 values were
corrected using wells containing 100 
µL of water.

### Bacitracin sensitivity assays.
The sensitivity of
B. subtilis
strains to bacitracin was determined as the MIC. For this, serial 2-fold dilutions of
Zn²⁺-bacitracin was added to final concentrations of 5, 10, or 30
µg mL⁻¹, with one well left uninduced. Cultures were further incubated
for 1 h. The OD₆₀₀ values were monitored every 5 min. OD₆₀₀ values were
normalized to cell density by dividing each data point by its correspond-
ing corrected OD₆₀₀ value (RLU/OD).

### Western blot analyses.
To compare production levels of BceB and its
dervatives in B. subtilis, strain SGB176 carrying plasmid
RESULTS
Identification of signaling-defective derivatives of BceB. In order to identify mutations that led to a loss of the signaling activity of the BceAB transporter, a plasmid (pER703) containing only the permease gene (bceB) under the control of the xylose-inducible promoter \( P_{\text{xyI}} \) was chosen. In a previous study, this construct had been described as leading to poor complementation of signaling in a bceB-deleted strain of \( B. \ subtilis \) (10). However, a subsequent more detailed characterization showed that the signaling activity in this strain was in fact high at very low bacitracin concentrations: it showed a maximal induction of the target promoter, \( P_{\text{bacI}} \), between 0.1 and 0.5 \( \mu \text{g m}^{-1} \) bacitracin (data not shown), whereas previous assays had been performed at 50 \( \mu \text{g m}^{-1} \) bacitracin, which is lethal to this strain and therefore led to the earlier observation of low signaling output (10). We attribute this phenotype to very weak expression of bceB due to the poor ribosome binding site of the gene, resulting in low levels of the transporter in the cell and thus in increased bacitracin stress and associated signaling at lower concentrations. This construct was ideally suited for the identification of loss-of-function mutations, because screening could be carried out at concentrations that are sublethal even for strains with defects in bceAB (MIC = 4 \( \mu \text{g m}^{-1} \); see below and Table 3).

Chemically mutagenized plasmid DNA of pER703 was used to transform strain TMB301, which carries a deletion of bceB and a \( P_{\text{bacI}}-\text{lacZ} \) reporter construct (10). After growth on indicator plates containing 0.5 \( \mu \text{g m}^{-1} \) bacitracin and X-Gal, colonies containing functional copies of bceB were dark blue, while those carrying defective constructs were white. We screened approximately 20,000 colonies, leading to the identification of 33 clones with an amino acid exchange causing a loss of signaling activity. Most constructs carried only a single point mutation, and some exchanges were obtained in multiple independent clones (three clones with A301T, two with M551I, and two with a mutation of S219 to F or P, respectively), indicating nearly complete saturation of the screen.

In total, 28 different amino acid exchanges were identified. A striking first observation was an accumulation of mutations in the C-terminal region (from position 523) of BceB, particularly in the eighth and tenth transmembrane helices (Fig. 1). To further characterize the obtained mutations, 26 amino acid exchanges were reconstructed individually using site-directed mutagenesis of plasmid pSG704. This construct contains the entire transporter operon \( bceAB \) under the control of \( P_{\text{bacI}} \) and shows much stronger complementation of both resistance and signaling activities in a \( bceAB \)-deleted strain than pER703 (see below). Importantly, the xylose-inducible promoter ensured equal \( bceAB \) expression levels for all of the mutants, irrespective of their signaling activity. Two mutations (T409I and S512L) could not be reconstructed despite several attempts and were therefore not analyzed further. Additionally, the serine residue at position 219 was replaced not only by Phe as in the original mutation but also by Cys to introduce a more conserved change (\(-\text{OH} \) by \(-\text{SH} \) group; see below). All 26 exchanges are summarized in Table 3.

Resistance behavior of mutant derivatives. Because the identification of mutations was based on their loss of signaling activity, it was first tested whether they were still able to confer bacitracin resistance or whether both functions of the transporter were affected. For this, the wild-type construct pSG704 and all derived mutant constructs were introduced into strain TMB035 (\( bceAB \); Kan). The MIC of TMB035 was determined as 4 \( \mu \text{g m}^{-1} \) bacitracin and that of SGB170 (\( bceAB \); Kan transformed with wild-type pSG704) as 32 \( \mu \text{g m}^{-1} \). It should be noted that the parental strain \( B. \ subtilis \) W168 has an MIC for bacitracin of 128 \( \mu \text{g m}^{-1} \) and that pSG704 does not fully complement the deletion of \( bceAB \). For the purpose of discussing the obtained mutations, the reduced MIC of

### Table 3 Summary of mutations analyzed in this study

<table>
<thead>
<tr>
<th>Amino acid exchange</th>
<th>MIC (( \mu \text{g m}^{-1} ))</th>
<th>Signaling phenotype</th>
<th>Interaction with BceB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A73T</td>
<td>8–16</td>
<td>Altered sensitivity</td>
<td>+</td>
</tr>
<tr>
<td>R83W</td>
<td>4–8</td>
<td>Altered sensitivity</td>
<td>+</td>
</tr>
<tr>
<td>G215R</td>
<td>8–16</td>
<td>Low signal output</td>
<td>+</td>
</tr>
<tr>
<td>S219F</td>
<td>4–8</td>
<td>Wild type like</td>
<td>+</td>
</tr>
<tr>
<td>S219C</td>
<td>4</td>
<td>No activity</td>
<td>ND</td>
</tr>
<tr>
<td>G247R</td>
<td>4</td>
<td>No activity</td>
<td>ND</td>
</tr>
<tr>
<td>A301T</td>
<td>8–16</td>
<td>Low signal output</td>
<td>+</td>
</tr>
<tr>
<td>S316L</td>
<td>4</td>
<td>Low signal output</td>
<td>+</td>
</tr>
<tr>
<td>G525D</td>
<td>4–8</td>
<td>No activity</td>
<td>+</td>
</tr>
<tr>
<td>G535E</td>
<td>4–8</td>
<td>Altered sensitivity</td>
<td>+</td>
</tr>
<tr>
<td>S542L</td>
<td>8–16</td>
<td>Altered sensitivity</td>
<td>+</td>
</tr>
<tr>
<td>L567P</td>
<td>32</td>
<td>Altered sensitivity</td>
<td>ND</td>
</tr>
<tr>
<td>C544Y</td>
<td>8</td>
<td>No activity</td>
<td>+</td>
</tr>
<tr>
<td>M551I</td>
<td>16</td>
<td>Low signal output</td>
<td>+</td>
</tr>
<tr>
<td>G609R</td>
<td>4–8</td>
<td>Altered sensitivity</td>
<td>ND</td>
</tr>
<tr>
<td>M616L</td>
<td>8–16</td>
<td>Wild type like</td>
<td>+</td>
</tr>
<tr>
<td>V619M</td>
<td>8–16</td>
<td>Low signal output</td>
<td>+</td>
</tr>
</tbody>
</table>

\( ^{a} \) The following mutations showed wild-type behavior: RQQ, V22M, A117T, V130I, G381D, V246L, H430Y, G593D, T624I, and S628P.

\( ^{b} \) Data represent MIC of bacitracin. MICs between two values showed variable results between biological triplicates; the MIC for a strain carrying the wild-type construct is 32 \( \mu \text{g m}^{-1} \) and for TMB035 (\( bceAB \); Kan) is 4 \( \mu \text{g m}^{-1} \).

\( ^{c} \) Categories of phenotypes as detailed in the text and shown in Fig. 2.

\( ^{d} \) As determined by bacterial two-hybrid assays (Fig. 4). +, interaction; –, no interaction; ND, not determined.
strain SGB170 is referred to as the “wild-type” resistance level throughout this article.

The strains carrying mutated complementation constructs fell into three groups of resistance phenotypes (Table 3). Seven mutations caused a (nearly) complete loss of resistance, as seen by MIC values of 4 or of 4 to 8 \( \mu g/\text{ml} \). Eight mutations retained a partial ability to confer resistance, with the resulting strains displaying MICs between 8 and 16 \( \mu g/\text{ml} \), and the remaining 11 mutations did not appear to affect resistance (MIC = 32 \( \mu g/\text{ml} \)).

Mapping these different categories of phenotypes onto the predicted transmembrane topology of BceB did not reveal any obvious correlation between the position in the protein and the severity of the mutation’s effect, apart from the general clustering of mutations in the C-terminal half of BceB as mentioned above (Fig. 1A).

**FIG 1** Distribution of amino acid exchanges in BceB by effects on resistance and signaling. The position of each amino acid exchange in the predicted transmembrane topology of BceB is marked by a star, and the exact exchange is given. Shading of stars is according to the effects of each mutation on bacitracin detoxification (A) or activation of signal transduction (B). Exchanges resulting in wild-type behavior are not shown. Details on the observed phenotypes are given in the text and Table 3. Topology prediction was carried out by MemBrain (30), and the results are displayed using TOPO2 (http://www.sacs.ucsf.edu/). The cytoplasmic membrane is indicated by horizontal lines, and the orientation of the schematic is given on the left.
were introduced into strain SGB079 that carries a deletion of bceAB and a reporter construct of the target promoter P_{bce} fused to luciferase. The strain harboring the wild-type construct showed only low basal expression of the promoter in the absence of bacitracin (ca. $2 \times 10^5$ RLU OD$^{-1}$), which was induced 10-fold in the presence of 3 g ml$^{-1}$ of bacitracin. Addition of 10 or 30 g ml$^{-1}$ bacitracin led to a strong upregulation of promoter activity, reaching ca. $6 \times 10^5$ and $1 \times 10^6$ RLU OD$^{-1}$, respectively, within 45 min postinduction (Fig. 2A, panel “WT-like”).

Analysis of the mutated constructs showed that their signaling phenotypes could be classified into four different categories. About half of the derived strains displayed behavior similar to that of the wild type, with maximum induction occurring after addition of 30 g ml$^{-1}$ bacitracin and reaching between 60% and 90% of the maximal activity compared to the wild type (Fig. 2B, panel “WT-like”). Six mutants also reached between 60% and 80% of the maximal activity, and yet they responded most strongly to the lowest concentration of 3 g ml$^{-1}$ bacitracin and had reduced activities at 10 or 30 g ml$^{-1}$ (Fig. 2, panels “Altered sensitivity”). Most of these strains also had low MIC values, and we speculate that this reduced ability to detoxify bacitracin may be the actual cause of the signaling “defects”: poor efficiency in removing the antibiotic would cause increased bacitracin stress at low concentrations, leading to an increase in signaling compared to the wild-type level. At concentrations above the MIC (i.e., 10 or 30 g ml$^{-1}$), growth inhibition and cell death would then cause an apparent reduction in promoter activation. Apart from the changed dose-response behavior, the overall output of this group of BceB derivatives was high (ca. 80% of the wild-type level; Fig. 2), suggesting that the signaling pathway itself was intact in these proteins. The third category of mutants displayed overall low levels of promoter induction, reaching only 20% to 30% of the wild-type level. Their dose-response behavior differed between strains (Fig. 2, panels “Low signal output”). The final group was comprised of four strains that were unable to induce expression of the reporter construct (Fig. 2, panels “No activity”). The apparent slight increase between 40 and 60 min postinduction is likely an experimental artifact derived from normalizing luminescence values to OD$_{600}$ values near zero, due to addition of lethal bacitracin concentrations. A comparison of signaling phenotypes to bacitracin resistance for each mutant is presented in Table 3.

To determine if there was a correlation between the position of the mutated amino acid in the protein and the observed phenotype, all mutations with an effect on signaling were mapped onto the mutated amino acid in the protein and the observed phenotypes could be classified into four different categories. With the exception of BceB carrying the G247R substitution, which was not detectable by Western blotting, all derivatives of BceB were produced at the same level as the wild-type protein (Fig. 3). Therefore, the observed phenotypes were direct effects of the amino acid exchanges and cannot be explained by altered protein expression.

### Protein–protein interactions between BceB and BceA or BceS

Because the signaling pathway within Bce-like modules involves both the transporter and the histidine kinase (10, 26) and because the permeases and histidine kinases were shown to have coevolved (9), direct interactions between the two proteins have been proposed. The first experimental evidence for this was obtained for a Bce-like system from S. aureus, using a bacterial two-hybrid assay (15). In order to test these interactions within the Bce module of B. subtilis, we fused BceB, BceS, and BceA to the T18 and T25 domains of bacterial adenylate cyclase (22) and tested these constructs for interaction. The optimal combination for interactions between the permease and ATPase components of the transporter was found to be BceA-T18 (pUT18-BceA) with T25-BceB (pKT25-BceB) (Fig. 4A and data not shown). Interaction between BceB and BceS could also be shown, with the strongest results obtained for T25-BceB paired with BceS-T18 (pUT18-BceS) (Fig. 4B and data not shown).

Two replacements, R83W and M551I, that both affected signaling and resistance are located in cytoplasmic loops of BceB (Fig. 1). It was therefore conceivable that they affected the interaction between permease and ATPase of the transporter. While there is no conservation in sequence or even location of the interface between permease and ATPase among the different types of ABC transporters, in all cases the interaction involves a small cytoplasmic alpha-helix of the permease (27, 28). Because the two cytoplasmic loops harboring the amino acids 83 and 551, respectively, possess predicted alpha-helical structures and are thus candidate regions for interaction, we introduced these two mutations into the two-hybrid plasmid pKT25-BceB and tested the constructs against BceA-T18. No differences compared to the wild-type constructs were observed (Fig. 4A), showing that the physical interaction between BceB and BceA was not affected. However, this does not exclude potential defects in the coupling of ATP hydrolysis to transport.

We next introduced mutations that caused a signaling defect into pKT25-BceB and tested for interaction with BceS. Again, most constructs showed the same blue coloration of colonies as seen with wild-type BceB, with the exception of G215R, which produced white colonies and was thus no longer able to interact with BceS (Fig. 4B). These results show that the majority of mutations did not abolish the physical interaction between the transporter and histidine kinase but rather affected signaling by more subtle changes.

### Genetic separability of signaling and resistance

Many of the mutations identified in this study affect signaling and resistance to...
FIG 2 Signal transduction activities. (A) Time courses of promoter induction after addition of Zn^{2+}-bacitracin (Bac; concentrations are given in the key) in strains harboring the P_{bceA-luxABCDE} reporter construct pSDlux101. Bacitracin was added to exponentially growing cultures at time point 0 min, and luminescence (relative luminescence units, RLU) and cell growth (optical density at 600 nm, OD) were measured in 5-min intervals. Luminescence was normalized to cell density and is expressed as RLU OD^{-1}. Example graphs for strains displaying the four observed categories of signaling phenotypes are shown ["WT-like," strain SGB082 carrying the wild-type sequence construct pSG704; "Altered sensitivity," strain carrying pSG704(R83W); "Low signal output," strain carrying pSG704(A301T); "No activity," strain carrying pSG704(C544Y)]. The arrows indicate the time (48 min postinduction) used to compare all mutated strains in panel B. (B) Dose-response behavior of all analyzed strains at 48 min postinduction. The example strains also shown in panel A are indicated by asterisks. Phenotype categories and labeling of symbols are as described for panel A. All data shown are the means ± standard errors of the means of the results determined with four to six biological replicates.
tained at a significant level (MIC \text{H11005} is still sufficient for signaling. The idea of a potential role of S219 in substrate binding or translocation might be supported by its location on the extracellular face of the cytoplasmic membrane (Fig. 1) in the vicinity of the cellular target of bacitracin, undecaprenyl pyrophosphate. Further studies on substrate binding and transport will be required to answer this.

As mentioned above, in a second clone of our initial screen an S219P mutation was identified, emphasizing the importance of this position. The alignment of BceB homologues shows that this position is commonly occupied by the small residue Ser or Ala (Fig. 5), whereas both isolated mutations caused changes to bulky amino acids. We therefore reconstructed the exchange with a replacement by Cys, which merely changes the hyroxyl side chain of Ser to a sulfhydryl group. Surprisingly, this replacement led to a complete loss of function (Table 3), possibly caused by oxidation of the Cys side chain (although BceB does not contain any other extracellular Cys residues that might form disulfide bridges with Cys219), and again supports the idea of the functional importance of position 219 in the transporter.

**DISCUSSION**

In this study, we chose a random mutagenesis approach to identify residues and regions in the permease of the bacitracin transporter BceAB that are involved in mediating resistance or signaling to the histidine kinase. Fifteen such positions were found. Mapping these amino acids onto the predicted transmembrane topology of BceB showed an accumulation of mutations in the C-terminal half of the protein, with six mutations clustering in transmembrane helix VIII and the adjacent cytoplasmic loop (Fig. 1). This corre-
lation becomes even more pronounced when the degree of conservation of each position is considered: four of the five mutations identified in the N-terminal half of the protein (up to transmembrane helix VI) affect highly conserved positions, i.e., A75, R83, G215, and G247 (Fig. 5). It is therefore likely that these amino acids have a structural role in the transporter and that no or little functional information can be obtained from their characterization. In particular, Gly residues within transmembrane helices, such as G215 and G247, are often important for helix packing in membrane proteins (29) and mutations are unlikely to be tolerated. Consistently, BceB carrying the exchange G247R was no longer produced (Fig. 3), and the mutation G215R abolished physical interaction with BceS (Fig. 4).

In contrast, only one of the six mutations located in transmembrane helix VIII and the adjacent cytoplasmic loop affected a highly conserved position, G525 (Fig. 5), and, again consistent with a structural role of this Gly residue, this mutation led to a complete loss of signaling and resistance activities (Table 3). The remaining five positions, G535, S542, C544, M551, and L567, are conserved only weakly or not at all (Fig. 5), and even drastic mutations such as Gly to Asp or Glu still allowed at least partial activity of the transporter (Table 3). This region of BceB is therefore clearly important for the specific functions rather than the global structure of BceB. Because most of these mutations affect both signaling and resistance, the low degree of conservation at these positions may point toward a role either in substrate specificity or

FIG 5 Multiple sequence alignment of BceB and homologs. The amino acids of BceB that were changed during mutagenesis are indicated by arrowheads, and their position numbers are given. The alignment was calculated using default settings of ClustalW implemented in BioEdit (31). Predicted transmembrane helices of BceB are marked by gray lines below the alignment. The poorly conserved region of the extracellular domain (residues 320 to 510 [BceB numbering]) was removed, as indicated by the diagonal black bars. Identical residues are shown as white letters on black fields and similar residues as black letters on a gray background. The threshold conservation for shading was 70%. Names of proteins are given on the left. BceB and PsdB are from *B. subtilis*, ABC09 is from *L. casei*, VraG, BraE, and VraE are from *S. aureus*, and EF2751 and EF2049 are from *E. faecalis* (see the text for references).
in communication with BceS. It is, however, also conceivable that the function of this transmembrane helix is the transmission of a signal within the transporter. The location of transmembrane helix VIII immediately adjacent to the large extracellular domain, which was shown to constitute the determinant of substrate specificity in BceB-like transporters from *S. aureus* (14), might well support the latter hypothesis, where extracellular binding of bacitracin may be communicated to the cytoplasmic face of the membrane or even further toward the C-terminal end of the protein. Interestingly, another cluster of mutations was found in the last transmembrane helix, and these also affect positions of low sequence conservation (Fig. 5). However, as these mutations caused differing defects in the transporter (Fig. 1 and Table 3), their functional role remains unclear. There is as yet no clear indication of where the interaction between BceB and BceS occurs. An extracellular interaction appears unlikely, because BceS contains only three predicted extracellular residues. Instead, an interaction either within the membrane or between cytoplasmic domains might be proposed. Such physical contacts could then transmit conformational changes from the transporter to the histidine kinase, causing changes in the autophosphorylation activities. Either potential interaction site could explain the accumulation of mutations in transmembrane helix VIII and the adjacent loop. However, with the current lack of in vitro data, such a model has to remain speculation.

Despite its large size of over 200 amino acids, no mutation could be identified in the extracellular domain of BceB. This is consistent with the very low degree of sequence conservation of this region across all BceB homologues identified to date, even among transporters that share bacitracin as a substrate (9). Our results therefore confirm the previously observed enormous freedom in sequence space of this domain, while it still retains full activity.

Several mutations led to nearly complete loss of both activities. These included all four mutations that were classified as “No activity” in the signaling assays (Fig. 2), which all were also unable to impart bacitracin resistance (MIC of 4 to 8 \( \mu \)g ml\(^{-1} \); Table 3). A similar effect, albeit with 20% to 30% residual signaling activity, was observed for mutations S316L and G215R (Fig. 2 and Table 3). These mutations therefore appear to cause global defects in the transporter. Of these, the mutation G215R presents an interesting situation: the maximal promoter activation obtained in signaling assays reached ca. 30% of wild-type levels (Fig. 2), and yet in bacterial two-hybrid assays this BceB derivative showed no interaction with BceS (Fig. 4). As discussed above, exchange of the highly conserved, membrane-located Gly residue for an Arg most likely introduced structural changes in the transporter, and these appeared to have strong effects on protein-protein interactions within the Bce module. However, the significant signaling activity observed suggests that while these interactions are sufficiently weakened to give negative results in the two-hybrid assay, the signal can still be passed to the histidine kinase. This is in contrast to the remaining mutations that inhibit signaling but all still allow a physical interaction in the two-hybrid analyses (Fig. 4 and Table 3). In these cases, the single amino acid exchange appears to be insufficient to destroy the protein-protein interactions but may rather affect the flow of information between BceB and BceS. Alternatively, the effects of the mutation may have no direct connection to the signaling process itself but may rather affect the transport process or transmission of conformational changes within the transporter as discussed above.

In the course of earlier studies on BceAB-like transporters, several systems could be identified that no longer possess the dual functions for signaling and resistance but have become specialized signaling or detoxification transporters. With the aim of identifying specific residues for one or the other function, examples for all three types of BceAB homologues were chosen for the multiple sequence alignment shown in Fig. 5. BceB and PsdB of *B. subtilis* as well as ABC09 of *L. casei* belong to dual-function transporters (10, 11, 17). VraG and BraE of *S. aureus* are the permeases of transporters that fulfill only a sensory function (14, 15), which is also the case for EF2751 of *Enterococcus faecalis* (our own unpublished results). As examples for transporters that mediate resistance but no signaling, the permeases VraE of *S. aureus* (14) and EF2049 of *E. faecalis* (our own unpublished data) were chosen. Comparison of these sequences and the positions identified as important in our mutagenesis screen did not reveal any patterns of sequence conservation that may be attributed to a particular role in either signaling or resistance.

Nevertheless, we were able to show at least partial genetic separability of the two traits. Three exchanges (A301T, M551I, and V619M) were associated with a strong reduction in signaling activity but retained the ability to mediate significant bacitracin resistance. One BceB derivative (S219F) displayed a complete loss of resistance but was still capable of signaling (Fig. 2 and Table 3). While the maximal signaling output was reduced to ca. 60% of the wild-type level, it should be noted that the bacitracin concentration used (30 \( \mu \)g ml\(^{-1} \)), was well above the MIC value of the strain (4 \( \mu \)g ml\(^{-1} \)), which may lead to an underestimation of the actual signaling activity of the transporter.

Furthermore, these results, particularly those from the three mutations mentioned above (A301T, M551I, and V619M), help to answer a long-asked question of the transporter’s role in signaling. It has been proposed that BceAB might in fact function as an importer rather than an exporter (10, 14). If this were the case, its sole function in signaling might then be to transport bacitracin to the cytoplasm where BceS might detect the antibiotic directly. This hypothesis can now be refuted: BceAB carrying one of these three mutations is still able to transport bacitracin at sufficient rates to impart resistance. Thus, if it actually imports bacitracin, resulting intracellular concentrations similar to those seen with the wild type would have to be expected, and accordingly, if BceS were a sensor of intracellular bacitracin, wild-type-like signaling output should be observed. Yet only very low signaling is observed, demonstrating that not BceS but the transporter itself, irrespective of the direction of transport, is indeed the sensory component of the module. Therefore, our findings not only show that signaling and resistance are not strictly coupled functions of BceB but also provide some valuable insights into the mechanism of these unique resistance modules.

In summary, we here showed that mutations that affect specific functions of BceAB are primarily found in the C-terminal half of the permease, particularly in transmembrane helix VIII. This region will therefore be a primary target for future investigations into the signaling and resistance mechanisms of this unusual transporter. Our results further demonstrate that while signaling and resistance are functionally interconnected, they are not strictly coupled processes, even in a transporter like BceAB that is capable of both activities.
ACKNOWLEDGMENTS

We thank Anna Staron for cloning of the bacterial two-hybrid constructs, Stefanie Zapf and Ina Lackerbauer for technical assistance, Richard Losick for supplying the vector pAH3328, and Thorsten Mascher and Ralf Heermann for critical reading of the manuscript.

This work was supported by grants from the Deutsche Forschungsgesellschaft (GE2164/3-1) and the Fonds der Chemischen Industrie.

F.K. performed the site-directed mutagenesis, characterized the mutants, and participated in cloning of constructs; S.D. performed the Western blot and two-hybrid analyses and participated in cloning of constructs and site-directed mutagenesis; R.S. performed the random mutagenesis; and S.G. designed the study, coordinated the experimental work, and wrote the manuscript.

REFERENCES