Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*

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Abstract

Peptide antibiotics are produced by a wide range of microorganisms. Most of them target the cell envelope, often by inhibiting cell wall synthesis. One of the resistance mechanisms against antimicrobial peptides are detoxification modules consisting of a two-component system and an ABC transporter. Upon detection of such a compound, the two-component system induces the expression of the ABC transporter, which in turn removes the antibiotic from its site of action, mediating resistance of the cell. Three such peptide antibiotic sensing and detoxification modules are present in *Bacillus subtilis*. Here we show that each of these modules responds to a number of peptides and confers resistance against them. BceRS-AB responds to bacitracin, plectasin, mersacidin and actagardine. YxdJK-LM is induced by a cationic antimicrobial peptide, LL-37. The PsdRS-AB (formerly YvcPQ-RS) system primarily responds to lipid II-binding lantibiotics, such as nisin and gallidermin. We characterized the *psdRS-AB* operon and defined the regulatory sequences within the P_{psdA} promoter. Mutation analysis demonstrated that P_{psdA} expression is fully PsdR dependent. The features of both the P_{bceA} and P_{psdA} promoters make them promising candidates as novel whole cell biosensors that can easily be adjusted for high-throughput screening.
Introduction

Peptide antibiotics are produced by a wide range of organisms and can be synthesized both ribosomally and non-ribosomally (22). Non-ribosomally synthesized antimicrobial compounds are mainly produced by bacteria and are often post-translationally modified (17). They can form linear polypeptides, such as gramicidin (25), or cyclic molecules, such as bacitracin and polymyxins (29, 36). Glycopeptides (e.g. vancomycin and ramoplanin) consist of a peptide backbone, which is further modified by glycosylation and methylation (12).

Ribosomally synthesized peptides, including lantibiotics and defensins, are more widespread and are produced by mammals, amphibians, insects, plants, and bacteria (17). They are often derived from small precursor peptides and are usually small (10-50 amino acids), with an overall positive charge and a significant number of hydrophobic residues (18).

Most peptide antibiotics target crucial steps in cell wall biosynthesis. The bacterial cell wall is a vitally important structure that gives the cell its shape, separates it from its environment, and acts as a molecular sieve (23). This makes it an important target for many antimicrobial compounds, which very often act by sequestering lipid II and by blocking transglycosylation and transpeptidation steps (47). Vancomycin, lantibiotics, ramoplanin and many defensins bind different moieties of lipid II (23, 45, 46). Vancomycin binds to the C-terminal Lys-D-Ala-D-Ala of the pentapeptide chain of the cell wall precursor (7). Nisin and nisin-like lantibiotics bind the pyrophosphate of lipid II, whereas the binding site of mersacidin and related lantibiotics includes both the MurNAc-GlcNAc sugar moiety and the pyrophosphate (10). Ramoplanin requires the presence of MurNAc-Ala-Glu pyrophosphate in order to bind to lipid II. Bacitracin inhibits a different step of cell wall biosynthesis by binding undecaprenyl pyrophosphate and inhibiting its dephosphorylation, thereby blocking its recycling and, ultimately, cell wall biosynthesis (42).
Because the production of peptide antibiotics is widespread, presence of an appropriate stress response system is necessary, both for the producer strains, as well as for those bacteria that are exposed to these compounds in their natural habitat. One type of detoxification systems against peptide antibiotics found mainly in Gram-positive bacteria are modules consisting of an ABC transporter, which is genetically and functionally linked to a two-component system (TCS) (23, 24, 30). Upon sensing the signal (i.e. presence of the antibiotic), the histidine kinase phosphorylates its cognate response regulator, which in turn induces the expression of the ABC transporter genes. The transporter facilitates removal of the antibiotic compound from its active site (23).

While few of these systems have been experimentally characterized to date, all respond to and mediate resistance against cell wall peptide antibiotics. In *Staphylococcus aureus*, the GraRS-VraFG system was found to respond to vancomycin, polymyxin B (34), gallidermin (19) and defensins (27). Homologous proteins mediate resistance to nisin in *Lactococcus lactis* (26) and to bacitracin in *Streptococcus mutans* (39, 51).

The genome of *B. subtilis* contains three such peptide sensing and detoxification (PSD) modules consisting of a TCS and an ABC transporter: BceRS-AB, YxdJK-LM, and YvcPQ-RS (Fig. 1A and 1B). BceRS-AB (PSD1) was initially identified as a bacitracin-specific detoxification module (31, 38). Recently, it has been shown to also respond to the defensin plectasin (46). The YxdJK-LM system (PSD2) responds to the human antimicrobial peptide LL-37 (40). The third system, YvcPQ-RS (PSD3), has been initially described as a part of bacitracin stress response network (31).

In this study, we aimed to identify novel inducers for all three PSD modules. Using disc diffusion assays and promoter-*lacZ* fusions, we screened a wide variety of cell envelope active compounds, including many peptide antibiotics. In addition, we performed a
comprehensive meta-analysis of all published stress response microarray datasets in order to identify additional inducers of bceAB, yvcRS and yxdLM expression.

We present evidence that the BceRS-AB system is not only a bacitracin-specific resistance determinant, but rather a PSD module that responds to a broader spectrum of compounds, including the lantibiotics mersacidin and actagardine, as well as the defensin plectasin. This module also mediates a certain level of resistance against these compounds.

For PSD3, it has been recently shown that the weak induction of the yvcR promoter by bacitracin is the result of cross-activation of the response regulator YvcP by the histidine kinase of the paralogous BceRS-AB system (42). In this study, we identified lipid II-binding peptides, mainly lantibiotics, but also one lipopeptide, enduracidin, as inducers of yvcRS expression. We further demonstrate that the ABC transporter YvcRS confers resistance against its inducers. Based on the primary inducers and the resistance profile, we renamed the yvcPQRS locus to psdRSAB (for peptide antibiotic sensing and detoxification).

Our data demonstrate that the P_bceA- and P_psdA-based reporter strains are sensitive and more specific biosensor for lipid II-binding peptide antibiotics than any of the established cell wall antibiotic biosensors currently available, such as the P_spmA- and P_lauA-derived reporter strains (32, 53). We provide evidence indicating that both biosensors could easily be modified to accommodate high-throughput screens for novel antimicrobial compounds using pure compounds, culture supernatant or even directly the producing strains.

Materials and methods

Bacterial strains and growth conditions. B. subtilis and Escherichia coli were routinely grown in Luria-Bertani medium (LB) at 37°C with agitation. For the induction of antibiotic production, B. subtilis ATCC6633 (a subtilin-producing strain) and B. subtilis W168 (sublancin-producing strain) were grown in Medium A (3). All strains used in this study are...
listed in Table 1. Ampicillin (100 µg/ml) was used for selection of plasmid pAC6 and its
derivatives in *E. coli*. Kanamycin (10 µg/ml) and chloramphenicol (5 µg/ml) were used for the
selection of the *B. subtilis* strains used in this study.

**Construction of transcriptional promoter-*lacZ* fusions.** All strains, plasmids and
oligonucleotides used in this study are listed in Table 1. Ectopic integrations of P<sub>psdA</sub>-*lacZ* and
P<sub>yxdL</sub>-*lacZ* fusions were constructed based on the vector pAC6 (49). Promoter fragments of
increasing lengths were generated by PCR. Standard cloning techniques were applied (44).
The inserts were verified by DNA sequencing. The resulting pAC6-derived plasmids (Table
1) were linearized with ScaI and used to transform *B. subtilis* with chloramphenicol selection.

**Promoter induction assays.** Screening for induction of P<sub>psdA</sub>, P<sub>bceA</sub> and P<sub>yxdL</sub> was done by disc
diffusion assays essentially as described previously (9). Briefly, the assays were carried out
using soft agar overlays of the reporter strains on LB plates containing 40 µg/ml X-Gal. Filter
paper disks carrying 5 µl of stock solution (antibiotics normally at the concentration of 100
mg/ml; Pep5 and mersacidin at 1 mg/ml, actagardine at 50 mg/ml, duramycin at 10 mg/ml,
subtilin and sublancin as 5 µl of overnight culture supernatant of *B. subtilis* ATCC6633 and *B.
subtilis* W168, respectively) were placed on top of the agar. The plates were incubated at
37°C. After incubation for 24 h, the plates were scored for the appearance of blue rings at or
near the edge of the zones of growth inhibition produced by diffusion of the antibiotics from
the filter disks.

For quantitative measurements of β-galactosidase activity, cells were grown in LB medium at
37°C with agitation until they reached an OD<sub>600</sub> of ~0.45. The culture was split, and an
inducing substance (at sublethal concentration; see Table 2) was added to one half, leaving the
other half untreated (uninduced control). Both cultures were incubated for 30 min at 37°C.
Cell pellets were resuspended in 1 ml of working buffer (20 mM β-mercaptoethanol, 60 mM
Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, pH 7.0) and assayed for β-galactosidase activity as described, with normalization to cell density (35).

**Determination of growth inhibition and the minimal inhibitory concentration (MIC).**

For concentration-dependent induction and killing experiments, cells were grown in LB medium to mid-log growth phase (OD$_{600}$ of ~0.45), and antibiotics were added to the cultures as indicated. An uninduced culture was used as a negative control. The cultures were incubated with agitation at 37°C. A sample was taken after 30 min for β-galactosidase assays (see above), and the turbidity of the remaining culture was measured for at least 4 h to monitor the concentration-dependent effects of the antibiotics on cell growth.

MIC assays were performed in Mueller-Hinton medium. Strains W168 (wild type), TMB035 ($bceAB$::kan), and TMB294 ($psdAB$::kan) were inoculated to an OD$_{600}$ of 0.05 and different concentrations of antibiotics were added to the medium. Cultures were incubated with agitation at 37°C for 6 hours before the determination of cell density. MIC was defined as the lowest concentration of antibiotic that fully inhibited the growth.

**Allelic replacement mutagenesis using LFH-PCR.** The long flanking homology PCR (LFH-PCR) technique is derived from a published procedure (55) and was performed as described previously (31). Briefly, a kanamycin resistance cassette was amplified by PCR using the vector pDG780 (15) as template. Two primer pairs were designed to amplify ~1,000-bp DNA fragments flanking the region to be deleted at its 5’ and 3’ ends. The resulting fragments are here called the “up” and “do” fragments. The 3’ end of the up fragment as well as the 5’ end of the do fragment extended into the gene to be deleted in a way that all expression signals of genes up- and downstream of the targeted genes remained intact. Extensions of ~25 nucleotides were added to the 5’ end of the up-reverse and the do-forward primers that were complementary (opposite strand and inverted sequence) to the 5’ and 3’ ends of the amplified resistance cassette. 100 to 150 ng of the up and do fragments and 250 to 300
ng of the kanamycin cassette were used together with the specific up-forward and do-reverse primers at standard concentrations in a second PCR reaction. In this reaction the three fragments were joined by the 25-nucleotide overlapping complementary ends and simultaneously amplified by normal primer annealing. The PCR products were directly used to transform *B. subtilis* W168. Transformants were screened by colony PCR, using the up-forward primer with a reverse check primer annealing inside the resistance cassette (see Table 1). The integrity of the flanking regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1,000 bp amplified from chromosomal DNA of the resulting mutants.

Results and Discussion

**Screen for inducers of bceAB, yxdLM and psdAB expression.** In order to identify specific inducers of the three detoxification modules of *B. subtilis*, we used reporter strains (Table 1) carrying a chromosomal pAC6-based transcriptional *lacZ*-fusion integrated at the *amyE* locus in the presence of an intact TCS-ABC system (Fig. 1B). Disc diffusion assays were used to screen a variety of peptide antibiotics and other cell envelope active compounds for their ability to induce β-galactosidase expression. In this assay, filter plates were placed on soft agar overlays on LB plates containing X-Gal. Bactericidal activity of a given antibiotic was visualized as the presence of a growth inhibition zone around the filter disc, and the promoter induction as a blue ring around the inhibition zone.

To gain a more comprehensive understanding of the inducer spectra, these *in vivo* studies were complemented with an *in silico* meta-analysis of a large panel of genome-wide expression profiles of *B. subtilis* after treatment with inhibitory compounds of different modes of action. This analysis included membrane active compounds, antibiotics targeting fatty acid biosynthesis, folate biosynthesis, cell wall biosynthesis, translation, DNA topology and
glycosylation, cationic antimicrobial peptides, and metal ions (9, 21, 31, 37, 40, 56) (see footnote to Table 2 for details). Of all compounds tested, only the peptide antibiotics listed in Table 2 acted as inducers of the three PSD modules. For comparison, the results obtained for an already established peptide antibiotic biosensor, based on P_{leuf}(32), are also listed.

**PSD1.** The BceRS-AB system was initially identified as a part of the bacitracin stress response network and an important bacitracin resistance determinant in *B. subtilis* (31, 38). Its expression was recently reported to be upregulated after treatment with a fungal defensin, plectasin (46). Using disc diffusion assays we found that expression of P_{bceA} is also strongly induced by two lipid II-binding lantibiotics, actagardine (formerly gardimycin) and mersacidin. We did not observe any induction after treatment with other lantibiotics, including nisin, sublancin and duramycin (Table 2). We also did not identify any other compound from the microarray meta-analysis that induced expression of P_{bceA}.

Bacitracin is a cyclic dodecylpeptide (Fig. 2) that binds undecaprenyl pyrophosphate and inhibits cell wall biosynthesis by preventing the recycling of this lipid carrier (48). The remaining three inducing compounds, plectasin, mersacidin and actagardine also inhibit cell wall biosynthesis, however by binding lipid II (5, 46). Actagardine and the closely related mersacidin belong to the class of lantibiotics with compact globular structures (57) (Fig. 2). Both compounds share a conserved structure that is predicted to form the lipid II-binding pocket (50) and bind the MurNAc-GlcNAc pyrophosphate (10). Plectasin is also thought to bind the pyrophosphate moiety of lipid II (46). Surprisingly, ramoplanin which has been predicted to possess a similar backbone fold to that of actagardine and mersacidin, and therefore a similar mechanism of action (10), does not induce the expression of the bceAB operon (Table 2). Therefore, the exact nature of the signal sensed by the PSD1 module BceRS-AB remains to be elucidated.
PSD2. For the YxdJK-LM module, we did not identify any novel inducers of \( P_{\text{yxdL}} \) (Table 2). The \( yxdL \) promoter has been previously described to respond to the cationic antimicrobial peptide LL-37 (40). This cathelicidin is produced by human neutrophiles and shows antimicrobial activity against both Gram-positive and Gram-negative bacteria (52). LL-37 was shown to induce the expression of a homologous system in \( S. \text{aureus} \) (27). As it is unlikely that a soil bacterium like \( B. \text{subtilis} \) responds specifically to a human neutrophil peptide, we suggest that the actual physiological inducer shares some chemical properties with LL-37, but remains yet to be identified.

PSD3. The PsdRS-AB system was initially described to respond to bacitracin (31, 38). We have recently shown that the weak bacitracin induction of the system is a result of cross-activation of PsdR by the paralogous BceRS-AB system (42). Using disc diffusion assays we found that \( P_{\text{psdA}} \) is induced by lipid II-binding lipopeptide, enduracidin, and lipid II-binding lantibiotics nisin, subtilin, actagardine and gallidermin. No induction was observed for other lantibiotics, including mersacidin, Pep5, sublancin and duramycin, as well as for lipid II-binding antibiotics of other classes, including vancomycin and ramoplanin (Fig. 1C, Table 2). The majority of inducers of \( P_{\text{psdA}} \) expression are cationic lipid II-binding lantibiotics, namely nisin, subtilin, and gallidermin (Fig. 2). They all share an N-terminal lipid II-binding motif, a so-called pyrophosphate cage (57). Actagardine, another inducing lantibiotic, differs from the above mentioned compounds as it lacks a positive charge (Table 2). However, it has been proposed that lantibiotics of this family require \( \text{Ca}^{2+} \) ions to obtain full activity and that these ions improve the interaction with the bacterial membrane by conferring a positive overall charge (6). Mersacidin, a lantibiotic closely related to actagardine, does not induce the \( psdAB \) expression. These two lantibiotics are highly similar (Fig. 2), but the activity spectrum of actagardine is different from that of mersacidin: while actagardine is most active against streptococci and displays low activity against staphylococci, mersacidin is most active against...
staphylococcal species (50), including MRSA (28). As actagardine induces \( P_{psdA} \) expression, whereas mersacidin does not, the PsdRS-AB system has to be able to distinguish between those two closely related compounds.

Enduracidin, a cyclic lipopeptide with a high level of similarity to ramoplanin (33) (Fig. 2), is the only inducer of \( P_{psdA} \) expression found in this study that is not a lantibiotic. Induction of \( psdAB \) expression by enduracidin was recently confirmed in an independent microarray study (43). The significance of this finding remains unclear, but the differential behavior of \( P_{psdA} \) for two pairs of very closely related compounds (mersacidin / actagardine and ramoplanin / enduracidin) strongly suggests that the Psd system responds to a very specific antimicrobial quality of these related compounds that goes beyond their known structural and/or functional features.

**Screen with lantibiotics-producing strains.** For the initial screen of inducing antibiotics, we used disc diffusion assays with pure compounds or supernatants of the lantibiotic-producing strains (Fig. 1C). Subsequently, we wanted to test if it is also possible to use the reporter strain for directly screening lantibiotic producers. Therefore, we streaked out the \( P_{psdA} \) reporter strain TMB299 on LB-X-Gal plates directly next to *B. subtilis* ATCC6633, which produces subtilin, a lantibiotic that in the disc diffusion assay induced \( P_{psdA} \) expression. The appearance of a blue color only on the side of TMB299 adjacent to the producer strain shows that it is possible to visualize the induction not only by pure substances, but also directly producer strains (Fig 1D). Because of their specificity and sensitivity, both the \( P_{psdA} \) and \( P_{bceA} \) reporter strain are promising candidates for the development of a whole cell-based biosensor for the identification of novel peptide antibiotics from compound libraries, culture supernatants or even directly from antibiotic-producing colonies (Fig. 1).

\( P_{psdA} \) and \( P_{bceA} \) are induced by cell wall antibiotics in a concentration-dependent manner. To further quantify our data, we analyzed the induction of the \( psdA \) and \( bceA \) promoters as a
function of the concentration of the inducing compound. To this end, we performed quantitative β-galactosidase assays for the inducing antibiotics (Fig. 3A and 4A). In the concentration-dependent induction experiments, cultures of the P_{psdA} and P_{bceA} reporter strains were grown to mid-log growth phase, and antibiotics were added to the cultures. After an induction for 30 min, a sample was taken for β-galactosidase assays. These assays not only confirmed all the compounds identified in the disc-diffusion assay as strong inducers (increase in induction ranging from 100-fold for P_{bceA} after induction with actagardine to 800-fold for P_{psdA} after treatment with the same lantibiotic), but also demonstrate a concentration-dependent induction of both promoters (Fig. 3A and 4A).

**PSD3.** A strong induction of the P_{psdA} promoter was observed already for 0.5 µg/ml nisin, and it reached its maximum at 4 µg/ml. A similar picture was obtained for subtilin, where 0.125% of *B. subtilis* ATCC6633 supernatant strongly induced the P_{psdA} promoter, with the highest induction observed after addition of 0.5% supernatant. Induction by gallidermin was visible from the concentration of 10 µg/ml, reaching its maximum at 100 µg/ml. Enduracidin induced P_{psdA} expression at much lower concentration (5 ng/ml), with the highest induction observed at 160 ng/ml (Fig. 3A). Actagardine was the strongest inducer of P_{psdA} expression, with the 800-fold induction observed at 10 µg/ml.

**PSD1.** The bceA promoter was induced by actagardine at 0.03 µg/ml, with the highest induction observed at 3 µg/ml (Fig. 4A). However, the induction was weaker than the one observed for the psdA promoter, reaching only ~100-fold. Bacitracin and mersacidin induced P_{bceA} to comparable levels, with the induction reaching its maximum at 100 µg/ml and 10 µg/ml, respectively (Fig. 4A).

At higher concentrations, a strong decrease in the β-galactosidase activity was observed for all lantibiotics tested, indicating cellular damage. Therefore, we also measured the turbidity of the remaining culture of the reporter strain used for β-galactosidase assay for at least 4 h.
postinduction to monitor the concentration-dependent effects of the antibiotics on cell growth. A rapid lysis was observed for the concentrations of antibiotics that led to a decrease in the β-galactosidase activity (Fig. 3B and 4B). These results demonstrate that cellular lysis interfered with the synthesis of β-galactosidase as has been observed for other antibiotic reporter strains before (32).

The ABC transporters BceAB and PsdAB confer resistance against compounds inducing their expression. As shown above, the psdA and bceA promoters are strongly induced by peptide antibiotics. The genes under control of these promoters, psdAB and bceAB, respectively, encode two subunits of ABC transporters, ATP binding protein and permease. For the PSD1 module it was previously shown that the ABC transporter BceAB mediates resistance against bacitracin (4, 42). This prompted the question if the same is true for the other inducing compounds, and if the paralogous ABC transporter PsdAB also confers resistance against its inducers. Therefore, we determined the corresponding minimal inhibitory concentration (MIC) values in Mueller-Hinton medium for the wild type and the isogenic transporter deletion mutants. As already indicated by the lysis curves (Fig. 3B/4B), the cultures often lyse rapidly after the addition of peptide antibiotics, but resume growth after a couple of hours, presumably due to turnover and degradation of the compounds. For the MIC determination, we therefore measured the OD_{600} after six hours of incubation (see Material and Methods). This time was sufficient for all antibiotics to develop their full inhibitory effect, while simultaneously being short enough to prevent that growth already resumed (data not shown).

The MIC of the psdAB mutant was reduced ~2-fold for nisin and subtilin, and ~8-fold for gallidermin, as compared to the wild type (Fig. 3C). These results demonstrate that the PsdAB transporter indeed confers resistance to all of the P_{psdA}-inducing compounds, presumably by acting as an ATP-driven peptide antibiotic-specific resistance pump.
Similar effects were observed for the bceAB mutant (Fig. 4C). The BceAB transporter confers a high level of bacitracin resistance (MIC of the bceAB mutant is reduced ~30 fold), as has been reported previously (31, 38). It also confers a more moderate level of resistance to actagardine and mersacidin (~2-4 fold). These results demonstrate that the BceAB transporter, like PsdAB, mediates resistance to a broader spectrum of peptide antibiotics. But in both cases, the efficiency of removal varies significantly between the different compounds, irrespective of the strength of induction (Figs. 3 and 4).

Identification of the minimal PsdR-dependent promoter region for the psdAB operon. In contrast to BceRS-AB system, the regulation of the paralogous PsdRS-AB module is poorly understood. The psdRSAB operon encodes a response regulator, a histidine kinase, and the two subunits of an ABC transporter (ATP binding protein and a permease), respectively (Fig. 1A). Two σ^A-dependent promoters can be identified in this locus, one upstream of yvcP (response regulator), and a second weak σ^A-dependent promoter upstream of yvcR (ATP binding protein). A potential transcriptional terminator downstream of yvcQ can be predicted, albeit with a low ΔG° value (-6 kcal mol⁻¹) (24). The expression of the yvcRS genes under inducing conditions was previously verified by Northern-blot (31), and the presence of a longer transcript yvcPQRS was also detected (24, 41). These results demonstrated a constitutive basal level of expression of the whole yvcPQRS operon, with a much higher expression of the yvcRS genes under inducing conditions (Fig. 1A).

The data presented so far, together with the knowledge gained from the BceRS-AB system, suggests that the response regulator PsdR is activated by its cognate histidine kinase PsdS in the presence of lantibiotics, binds to its operator sequence in the psdA promoter region, resulting in a strong induction of psdAB expression and therefore lantibiotic resistance (Fig. 1B). To verify this hypothesis, we analyzed the regulatory elements upstream of the inducible psdAB operon in more detail.
A -10 consensus sequence for σ^A can be predicted upstream of the psdA gene. No clear -35 sequence can be found at the appropriate position from the -10 sequence (Fig. 5A). Such a situation is often found in promoters regulated by transcriptional activators. Response regulators that act as transcriptional activators usually bind DNA via short binding sites (inverted or direct repeats) a few nucleotides upstream of the -35 promoter element (54).

To localize the binding site of the response regulator, we used a lacZ-based promoter deletion approach. Progressively shorter fragments of P_{psdA} (all ending at position +30 relative to the ATG start codon of psdA) (Fig. 5B) were used to generate pAC6-based transcriptional lacZ reporter fusions integrated at the amyE locus. Strains were grown until mid-log phase and treated with nisin (2 µg/ml). All constructs had very low lacZ expression in the uninduced state (Fig. 5C). In cells containing reporter fusions that included at least 105 bp of the upstream psdA promoter region, β-galactosidase activity was strongly induced by the addition of nisin to the medium, while a fragment extending to -94 showed no induction (Fig. 5C).

Next, we demonstrated the PsdR-dependence of P_{psdA}-induction by lantibiotics in an psdR deletion mutant by repeating the β-galactosidase assays with strains carrying the P_{psdA} reporter fusion in the presence of an intact PsdRS-AB system (TMB299) and in the psdR deletion background (TMB652): Nisin-dependent induction was completely abolished in the psdR deletion mutant (133 ± 11 and 0,19 ± 0,05 Miller Units in the wild type and psdR mutant, respectively). Therefore, induction of the psdAB genes in the presence of lantibiotics is completely PsdR-dependent.

Based on the results from the promoter deletion experiments we identified an eight-nucleotide imperfect (two mismatches) inverted repeat with a four-nucleotide spacing region (ATGTGACAgcatTGTAAGAT) at position -99 to -70 (Fig. 5A) as a possible site for DNA binding by PsdR. This binding sequence bears similarity to the operator upstream of P_{bceA} in the paralogous BceRS-AB module and has also been predicted in a comprehensive
bioinformatic analysis of response regulator-specific binding sites in low-GC Gram-positive bacteria (11). Moreover, the specific binding of PsdR to this DNA region has been demonstrated by DNase footprint experiments (14). Taken together, these results demonstrate that PsdR binds to the inverted repeat upstream of the P_{psdA} promoter and activates the expression of psdAB operon in the presence of suitable inducers.

Conclusions and Outlook

The aim of the present study was to identify inducers of the three PSD modules BceRS-AB (PSD1), YxdJK-LM (PSD2), and PsdRS-AB (PSD3) of B. subtilis. By combining an in silico meta-analysis of available microarray datasets with disk diffusion assays in vivo, we screened a wide range of antimicrobial compounds, including a number of peptide antibiotics. Using a promoter-lacZ fusion strain we identified lipid II-binding lantibiotics as the main group of inducers of P_{psdA} expression. We also identified two closely related lantibiotics, actagardine and mersacidin, as novel inducers of bceAB expression, which was previously thought to be a bacitracin-specific resistance pump (31, 38). In contrast, we were not able to identify novel inducers of P_{yxdL}.

Induction of psdAB expression is completely dependent on the response regulator PsdR. We characterized the minimal P_{psdA} promoter and identified an inverted repeat that is necessary for the PsdR-dependent P_{psdA} induction and presumably represents the PsdR binding site. Moreover, we demonstrated that the genes psdAB, which encode an ABC transporter, confer resistance against this group of lantibiotics. Therefore, both the PsdRS-AB and the BceRS-AB systems constitute stand-alone PSD modules.

**Inducer specificity.** The most puzzling result, and an important open question that remains to be answered, concerns the nature of the true stimuli sensed by both PSD modules. Both systems have a more specific inducer range of lipid II-interfering peptide antibiotics,
compared to the P_{liaI}-based biosensor (Table 2). Strikingly, on the one hand they can
discriminate between highly similar compounds, such as the mersacidin/actagardine or
enduracidin/ramoplanin pairs (PSD1 and PSD3; Fig. 2), while on the other hand they are also
able to respond even to compounds as different as bacitracin and plectasin (PSD1).

Nevertheless, all compounds belong to the group of peptide antibiotics that seem to share a
common cellular target: They all require the pyrophosphate moiety of the lipid carrier of cell
wall biosynthesis, undecaprenyl pyrophosphate, as a docking interface to exhibit their
antimicrobial activity (7, 47). While most of the compounds identified in our screen as
inducers of PSD1 and PSD3 activity bind to lipid II, bacitracin directly binds undecaprenyl
pyrophosphate, the subsequent intermediate of the lipid II cycle (48).

The second surprising result is the apparent lack of correlation between the strength of
induction and the rate of resistance conferred by the induced ABC transporters. Actagardine is
the strongest inducer of the PsdRS-AB system. Yet, the corresponding ABC transporter does
not confer any detectable resistance. On the other hand, PsdAB confers significant resistance
against gallidermin, despite 10-fold lower induction level (Fig. 3). Similarly, mersacidin is as
potent an inducer of bceAB expression as bacitracin. Yet, the degree of resistance is almost an
order of magnitude lower for mersacidin compared to bacitracin (Fig. 4).

**PSD modules as novel biosensors.** Bacterial reporter strains based on antibiotic-inducible
promoters are an efficient tool for detecting novel bioactive compounds. The well-defined
regulatory responses of *Bacillus subtilis* to different types of (antibiotic) stresses and the ease
of genetic manipulations make this bacterium a preferred model organism for studying the
mode of action of antibiotics by transcriptomics, proteomics and whole-cell-based biosensors
(1, 2, 20, 21, 53, 56).

Two comprehensive studies identified sets of *B. subtilis* promoters responding to antibiotics
interfering with major biosynthetic pathways (i.e. biosynthesis of DNA, RNA, proteins, the
cell wall, and fatty acids) or specific classes of antibiotics (20, 53). Each of these promoters responds to a wide range of compounds that affect the respective pathways. But some of the identified promoters either have a relatively high basal expression level or are only moderately induced (three- to tenfold). Therefore, the noise to signal ratio (and hence robustness) of these biosensors is not always ideal. A biosensor based on the P_{lant} promoter is both more specific and much more robust (32). It has a very low background activity, and is induced 50-500-fold in the presence of compounds interfering with the lipid II cycle of cell wall biosynthesis, such as bacitracin, ramoplanin and vancomycin (Table 2). This biosensor strain has recently been adapted for high-throughput screens in microtiter plate bioassays (8), again demonstrating the potential of whole-cell biosensors for large scale screens of novel antimicrobial compounds.

Whole-cell biosensors suitable for high-throughput screening need to be compound- or pathway-specific, robust and sensitive (13). Based on the data presented in this study, the P_{psdA} and P_{bceA} reporter strains fulfill these criteria. They are based on an established organism, have very low intrinsic activities and are strongly (more than 100-fold) induced by a small set of peptide (l)antibiotics that bind lipid II (Fig. 2). We have demonstrated that they can be applied to analyze purified compounds, culture supernatants, or the producing strains directly (Fig. 1, 3, 4 and Table 2). Despite our current lack of exactly defining the nature of the stimuli sensed by the PSD modules, our data indicates that the combined use of the reporter strains derived from PSD1 and PSD3 represent useful additions to the pool of B. subtilis biosensors currently available. They allow the identification of different, but related subsets of peptide antibiotics that bind the pyrophosphate moiety of the lipid carrier of cell wall biosynthesis. Such biosensors will be very beneficial for screening strain collections and compound libraries, given the large potential of peptide antibiotics as an addition/alternative to established antibiotics currently in clinical use.
Acknowledgements

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References


Table 1. Strains, plasmids and oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Strain, plasmid or nucleotide</th>
<th>Characteristics or description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>F’ endA1 hsdR17(qc mc”) glv44 thi-1 recA1 gyrA (Nal') relA1 ΔlacZYA-argF]U169 deoR[Φ80 lacIΔlacZ]M15</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W168</td>
<td>wild type, trpC2</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>ATCC6633</td>
<td>wild type, subtilin producer</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>TMB035</td>
<td>W168 fceAB::kan</td>
<td>(42)</td>
</tr>
<tr>
<td>TMB279</td>
<td>W168 amyE::(cat, P_psdA-122-82_lacZ)</td>
<td>(42)</td>
</tr>
<tr>
<td>TMB294</td>
<td>W168 psdAB::kan</td>
<td>(42)</td>
</tr>
<tr>
<td>TMB299</td>
<td>W168 amyE::(cat, P_psdA-110-30_lacZ)</td>
<td>(42)</td>
</tr>
<tr>
<td>TMB413</td>
<td>W168 amyE::(cat, P_psdA-105-30_lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td>TMB414</td>
<td>W168 amyE::(cat, P_psdA-104-30_lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td>TMB598</td>
<td>W168 amyE::(cat, P_psdA-103-37_lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td>TMB652</td>
<td>W168 amyE::(cat, P_psdA-110-30_lacZ) psdR::kan</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAC6</td>
<td>lacZ fusion vector, integrates at amyE, chloramphenicol resistance</td>
<td>(49)</td>
</tr>
<tr>
<td>pDF602</td>
<td>pAC6 P_psdA-105-30_lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pDF603</td>
<td>pAC6 P_psdA-104-30_lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pER605</td>
<td>pAC6 P_psdA-110-30_lacZ</td>
<td>(42)</td>
</tr>
<tr>
<td>pPH601</td>
<td>pAC6 P_yxdL-194-57_lacZ</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpsdA+30</td>
<td>AGTCGAGATCCGCCGATAGGTTCTGGTTTGCAACACG</td>
<td></td>
</tr>
<tr>
<td>PpsdA-105</td>
<td>AGTCGAATTCGTGAATGTGACGATTGTAAG</td>
<td></td>
</tr>
<tr>
<td>PpsdA-94</td>
<td>AGTCGAATTCACAGCTTGTAAGATTGGG</td>
<td></td>
</tr>
<tr>
<td>PysxL+57</td>
<td>GCATGGATCCAGGACACTTGGCTTATAGG</td>
<td></td>
</tr>
<tr>
<td>PysxL-194</td>
<td>GCATGAATTCCTCCCGGGTAAAGGACATC</td>
<td></td>
</tr>
<tr>
<td>psdR-up-fwd</td>
<td>CAAAAAGAGAGCTATGGCG</td>
<td></td>
</tr>
<tr>
<td>psdR-do-fwd</td>
<td>CCTATCACCCTAAATGGTGGCGCTGAAGGACAAATCCGATACACG</td>
<td></td>
</tr>
<tr>
<td>psdR-do-rev</td>
<td>CAGGGCCATCACCAATGGTAGATAGCGGAAGGATGAGCCGAAATG</td>
<td></td>
</tr>
<tr>
<td>Kan-fwd</td>
<td>GAAAAACAGATGCTACATAC</td>
<td></td>
</tr>
<tr>
<td>Kan-rev</td>
<td>CAGGGCCATCACCAATGGTGAGGTAAG</td>
<td></td>
</tr>
<tr>
<td>Kan-check-fwd</td>
<td>CGATACAAATTCCTGTAGGCCTCCGTCGG</td>
<td></td>
</tr>
<tr>
<td>Kan-check-rev</td>
<td>CATTCCCAACTCTTCATTCATCC</td>
<td></td>
</tr>
</tbody>
</table>

1 The positions of the cloned fragments are given relative to the “A” of the start codon of the corresponding gene.
2 Sequences are given in the 5’ → 3’ direction. Restriction sites are underlined. Sequences highlighted in bold italics are inverse and complementary to the 5’ (up-rev) and 3’ (do-fwd) end of the kanamycin cassette, respectively.
Table 2. Inducers of \( \text{P}_{\text{psdA}}, \text{P}_{\text{bceA}}, \text{P}_{\text{yxdL}} \) and \( \text{P}_{\text{liaI}} \) expression\(^a\).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Class</th>
<th>Char</th>
<th>Lipid II- binding(^b)</th>
<th>Pore forming(^b)</th>
<th>Conc.(^c)</th>
<th>( \text{P}_{\text{psdA}} )(^d)</th>
<th>( \text{P}_{\text{bceA}} )(^d)</th>
<th>( \text{P}_{\text{yxdL}} )(^d)</th>
<th>( \text{P}_{\text{liaI}} )(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacitracin</td>
<td>Cyclic peptide</td>
<td>+2</td>
<td>-</td>
<td>-</td>
<td>50 µg/ml</td>
<td>+++ (42)</td>
<td>+ (42)</td>
<td>-</td>
<td>+++ (31)</td>
</tr>
<tr>
<td>Enduracidin</td>
<td>Cyclic</td>
<td>+4</td>
<td>+</td>
<td>-</td>
<td>0,025 µg/ml</td>
<td>-</td>
<td>+++ (43)</td>
<td>-</td>
<td>+++ (43)</td>
</tr>
<tr>
<td>Ramoplanin</td>
<td>Cyclic lipopeptide</td>
<td>+2</td>
<td>+</td>
<td>-</td>
<td>5 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++ (32)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Glycopeptide</td>
<td>+2</td>
<td>+</td>
<td>-</td>
<td>2 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++ (9)</td>
</tr>
<tr>
<td>LL-37 Cathelicidin</td>
<td>Cyclic lipopeptide</td>
<td>+6</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>++ (40)</td>
<td>-</td>
</tr>
<tr>
<td>Plectasin</td>
<td>Defensin</td>
<td>+2</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>+++ (46)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actagardine</td>
<td>Lantibiotic</td>
<td>-1</td>
<td>+</td>
<td>-</td>
<td>3-10 µg/ml</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Duramycin</td>
<td>Lantibiotic</td>
<td>+1</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Gallidermin</td>
<td>Lantibiotic</td>
<td>-1</td>
<td>+</td>
<td>-</td>
<td>100 µg/ml</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++ (8)</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>Lantibiotic</td>
<td>+2</td>
<td>+</td>
<td>-</td>
<td>10 µg/ml</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nisin</td>
<td>Lantibiotic</td>
<td>+5</td>
<td>+</td>
<td>+</td>
<td>2 µg/ml</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++ (32)</td>
</tr>
<tr>
<td>Subtilin</td>
<td>Lantibiotic</td>
<td>+2</td>
<td>+</td>
<td>+</td>
<td>0.25% (\text{f})</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++ (8)</td>
</tr>
</tbody>
</table>

\(^a\) All inducers are peptide antibiotics. Based on the meta-analysis of transcriptome datasets and our disk diffusion screen, the following compounds do not induce \( \text{P}_{\text{psdA}}, \text{P}_{\text{bceA}}, \text{P}_{\text{yxdL}} \) and \( \text{P}_{\text{liaI}} \): cell wall biosynthesis inhibitors (amoxicillin, cefalexin, cephalosporin, cepotaxime, cefotaxime, dirampicillin, d-cycloserine, fruimicin, oxacillin, penicillin G, Pep5, PG-1, phosphomycin, ristocetin, sublancim 168, tunicamycin, membrane-active compounds and ionophors (gramicidin A, monensin, nigericin, nitrofurantoin, polymyxin B, polymyxin L-lysine, Triton X-114), compounds interfering with DNA topology (ciprofloxacin, coumermycin, metoxiloxacin, nalidixic acid, norflaxacin, novobiocin, fatty acid biosynthesis inhibitors (triclosan, cerulenin), folate biosynthesis inhibitors (dapsone, sulfacetamide, sulfamethizole, trimethoprim), inhibitors of protein biosynthesis (chloramphenicol, clarithromycin, clindamycin, erythromycin, fusidic acid, neomycin, puromycin, spectinomycin, tetracycline), metal ions (Ag(I), Cd(II), Cu(II), Ni(II), Zn(II), As(V), miscellaneous (actinomycin, azaserine, doxorubicin, ethidium bromide, HCP, rifampicin).

\(^b\) Based on the BACTIBASE database (http://bactibase.pfba-lab-tun.org/) (16)

\(^c\) \(\%\) \(\text{B. subtilis ATCC6633 supernatant}\)

\(^d\) All \(\text{P}_{\text{psdA}}, \text{P}_{\text{bceA}}, \text{P}_{\text{yxdL}} \) and \( \text{P}_{\text{liaI}} \) expression levels were determined using the \(\beta\)-galactosidase assay.

\(^e\) Based on the \(\beta\)-galactosidase assay.

\(^f\) Concentration resulting in the highest level of induction in quantitative \(\beta\)-galactosidase assay.

\(\%\) \(\text{B. subtilis ATCC6633 supernatant}\)
Figure legends

**Fig. 1.** Organisation of the *psdRS-AB* and *bceRS-AB* loci and induction by peptide antibiotics.

(A) Graphic representation of the *psdRSAB* and *bceRSAB* loci. Genes belonging to the *psd* and *bce* loci are shown in blue (two-component system) and green (ABC transporter); the genes flanking both operons are white. Promoters are marked with bent arrows, putative terminators are represented by vertical bars and a circle. (B) Regulatory principle and genetic setup of the Psd- and Bce-biosensor strains. The response regulator (PsdR or BceR), activated by the sensor kinase (PsdS or BceS), binds to its target promoter, and induces the expression of the ABC transporter encoding operons *psdAB* or *bceAB* (detoxification) and *lacZ* (production of β-galactosidase). (C) Example of a qualitative β-galactosidase assay with nisin, actagardine, mersacidin and gramicidin (disc diffusion assay). The reporter strain carrying a chromosomal *P_{psdA}-lacZ* fusion was used in soft agar overlays on LB plates containing X-Gal. Bactericidal activity is visualized as the presence of a growth inhibition zone around the filter disc, and the *P_{psdA}-dependent induction as a blue ring around the inhibition zone. (D) Qualitative β-galactosidase assay with the subtilin producer strain *B. subtilis* ATCC6633. The *P_{psdA}-lacZ* reporter strain TMB299 was streaked out on LB-X-Gal plates directly next to the *B. subtilis* ATCC6633. Appearance of a blue zone in the reporter strain next to the subtilin-producing strain shows the induction of *P_{psdA}*. 

**Fig. 2.** Schematic structures of peptide antibiotics inducing the Psd and Bce systems. Amino acids are represented by labelled grey circles. Charged amino acids are highlighted black (positive charge) or white (negative charge). Abu – aminobutyric acid, Chp – 3-chloro-4-hydroxyphenylglycine, Cit – citrulline, Dha – dihydroalanine, Dhb – 2,3-didehydrobutyrine, Dpg – 3,5-dichloro-4-hydroxyphenylglycine, End – Enduracididine, HAsn – β-hydroxyasparagine, Hpg – hydroxyphenylglycine, Man – Mannose, Orn – ornithine, aThr – *allo*-Threonine. Induction of the Bce/Psd system is indicated after the name B+/P+. 

25
Fig. 3. Concentration-dependent induction of $P_{psdA}$, lysis curves and minimal inhibitory concentrations of $B. subtilis$ cultures treated with actagardine, enduracidin, gallidermin, nisin, or subtilin (supernatant of the $B. subtilis$ ATCC6633 strain). (A) $\beta$-Galactosidase activities, expressed as Miller Units, of the $P_{psdA}$-lacZ reporter strain TMB299 induced with the above compounds. A log-scale is applied on the $y$-axis for reasons of clarity, due to the high dynamic range of $\beta$-galactosidase activities. (B) Concentration-dependent killing of TMB299. The times of antibiotic addition are indicated by arrows. The concentrations of actagardine, gallidermin, nisin (in $\mu$g/ml), enduracidin (in ng/ml), and subtilin (in % of $B. subtilis$ ATCC6633 supernatant) that affect the growth of $B. subtilis$ are indicated. (C) Minimal inhibitory concentration (MIC) assays for $B. subtilis$ cultures treated with nisin, subtilin and gallidermin. Wild type (●) and $psdAB$ deletion mutant (○) strains were inoculated to an OD$_{600}$ of 0.05 in Mueller-Hinton medium with different concentrations of antibiotics. Cultures were incubated with agitation at 37°C for 6 hours before the determination of cell density (OD$_{600}$). The MIC was defined as the lowest concentration of antibiotic that fully inhibited the growth.

Fig. 4. Concentration-dependent induction of $P_{bceA}$, lysis curves, and minimal inhibitory concentrations of $B. subtilis$ cultures treated with actagardine, bacitracin, and mersacidin. (A) $\beta$-Galactosidase activities, expressed as Miller Units, of the reporter strain TMB279 induced by actagardine, bacitracin, or mersacidin. A log-scale is applied on the $y$-axis for reasons of clarity, due to the high dynamic range of $\beta$-galactosidase activities. (B) Concentration-dependent killing of $B. subtilis$. The times of antibiotic addition are indicated by arrows. The concentrations of actagardine, bacitracin, or mersacidin (all in $\mu$g/ml) that affect the growth of $B. subtilis$ are indicated. (C) Minimal inhibitory concentration assay for the wild type (●) and isogenic $bceAB$ deletion mutant (○). See legend for Fig. 3C for experimental details.
Fig. 5. Functional analysis of the psdA promoter. (A) Intergenic sequence between psdS and psdA. All features are marked underneath the respective lines of the sequence. The end of psdS and the start codon of psdA are indicated below the sequence. The -10 P<sub>psdA</sub> promoter fragment and the putative ribosome binding site are denoted by -10 and RBS, respectively. The inverted repeat sequence of the putative PsdR binding site is boxed. The 5' ends of the fragments used for the promoter deletion analysis are labeled according to their position relative to the psdA start codon. The minimal promoter fragment for nisin-dependent induction is underlined. (B) Graphical representation of the intergenic region and the fragments used for the promoter deletion analysis. The features of the region are represented by black boxes and labeled as above. (C) β-Galactosidase assay for the promoter deletion analysis. Black bars indicate the uninduced control sample for each strain, and grey bars represent the sample induced with nisin.