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Mini*Bacillus* PG10 as a Convenient and Effective Production Host for Lantibiotics

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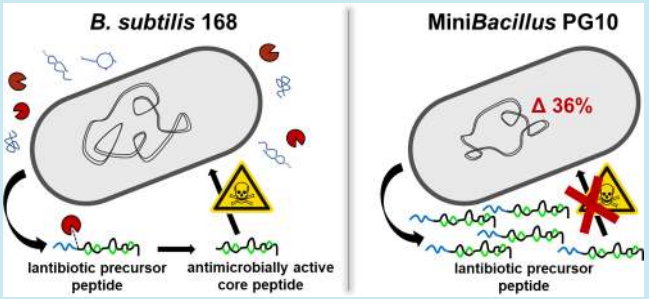
ABSTRACT: Efficient bacterial cell factories are important for the screening and characterization of potent antimicrobial peptides such as lantibiotics. Although lantibiotic production systems have been established in *Lactococcus lactis* and *Escherichia coli*, the industrial workhorse *Bacillus subtilis* has been left relatively unexplored as a lantibiotic production host. Therefore, we tested different *B. subtilis* strains for their ability to produce lantibiotic peptides by using the subtilin modification and transport enzymes derived from the natural subtilin producer *B. subtilis* ATCC 6633. Our study shows that although *B. subtilis* ATCC 6633 and 168 are able to produce various processed lantibiotic peptides, an evident advantage of using either the 8-fold protease-deficient strain WB800 or the genome-minimized *B. subtilis* 168 strain PG10 is the lack of extracellular serine protease activity. Consequently, leader processing of lantibiotic precursor peptides is circumvented and thus potential toxicity toward the production host is prevented. Furthermore, PG10 provides a clean secondary metabolic background and therefore appears to be the most promising *B. subtilis* lantibiotic production host. We demonstrate the production of various lantibiotic precursor peptides by PG10 and show different options for their *in vitro* activation. Our study thus provides a convenient *B. subtilis*-based lantibiotic production system, which facilitates the search for novel antimicrobial peptides.



KEYWORDS: *miniBacillus, lantibiotics, microbial cell factory, expression systems, extracellular serine proteases*

Due to the global problem of antibiotic resistant bacteria there is an increasing interest in novel bioactive natural compounds. A promising source is constituted by ribosomally synthesized and post-translationally modified peptides (RiPPs) which display a broad variety of bioactivities.[1] Within the RiPPs, the class of lantibiotics is of great interest because of their therapeutic potential and the possibility to use them as a food preservative. Lantibiotic peptides are naturally produced mainly by Gram-positive bacteria and harbor dehydrated residues and (methyl)lanthionine rings, of which the latter is important for their antimicrobial activity to a broad range of particularly Gram-positive bacteria.[2,3]

In the case of class I lantibiotics, the lantibiotic gene cluster generally consists of a structural gene (*lanA*), genes encoding the modification enzymes (*lanB* and *lanC*), an ATP binding cassette (ABC) transporter (*lanT*), a leader protease (*lanP*), regulatory elements (*lanRK*) and proteins providing immunity to the producer organism (*lanFEG* and *lanI*).[4,5] After ribosomal synthesis, the lantibiotic precursor peptide is composed of a leader and a core peptide of which the N-terminal leader peptide is important for guiding the precursor peptide to the modification, transport, and leader protease enzymes and for keeping the core peptide inactive during maturation. After leader peptide cleavage, generally by a specific protease, the mature antimicrobially active core peptide is released.

Although genome mining approaches greatly facilitate the identification of lantibiotic peptides, further characterization can be hampered due to difficulties in finding the right conditions for expression of the lantibiotic peptide by the natural microbial producer. Since chemical synthesis of lantibiotics has proven to be quite challenging, heterologous production and modification by a suitable microbial host in most cases enables researchers to obtain sufficient amounts for further investigation of potent lantibiotic peptides and their derivatives.[6] Thanks to the heterologous expression systems that were established in *Lactococcus lactis* and *Escherichia coli*, various novel lantibiotic peptides have been discovered and characterized.[7−10] Nevertheless, some evident obstacles have been encountered when using these production platforms. For instance, a disadvantage of using *L. lactis* as a heterologous

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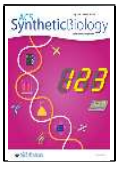

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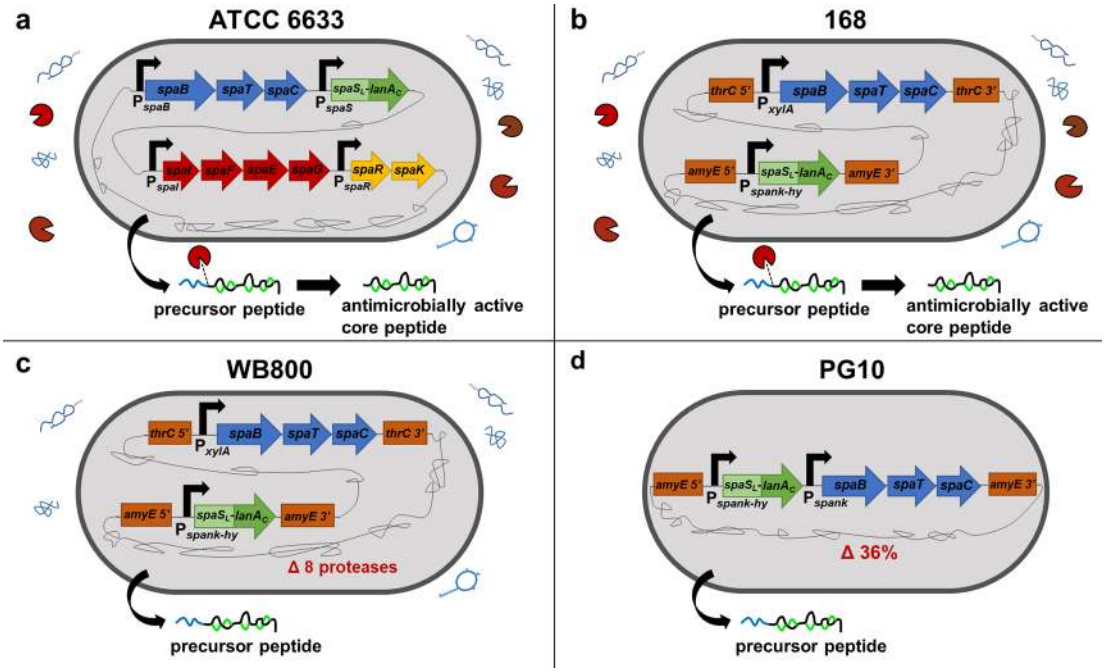

Figure 1. Overview of lantibiotic production platforms in different *B. subtilis* host strains. The genetics of the different lantibiotic expression systems and relevant characteristics of each *B. subtilis* host strain are shown. Extracellular proteases and native bioactive peptides (e.g., sublancin, surfactin, and subtilosin) of *B. subtilis* are visualized as red split circles and blue structures, respectively. (a) Lantibiotic production system in *B. subtilis* ATCC 6633 in which the *spaS* gene in the subtilin gene cluster is replaced by a gene encoding a hybrid peptide composed of the subtilin leader ($spaS_L$; containing the NisP cleavage site "ASPR") followed by a core peptide of interest ($lanA_C$). The expression systems in *B. subtilis* 168 (b) and WB800 (c) contain *spaBTC* controlled by the xylose-inducible promoter (P_{xylA}), and the lantibiotic structural gene encoding a hybrid peptide composed of the subtilin leader peptide and a core peptide of interest regulated by the IPTG-inducible hyper-spank promoter ($P_{spank\text{-}hy}$) integrated in the *thrC* and *amyE* locus, respectively. (d) Although three different expression systems were tested in mini*Bacillus* PG10, the expression system in which both the structural gene and *spaBTC* are integrated in the *amyE* locus and controlled by IPTG-inducible promoters ($P_{spank\text{-}hy}$ and P_{spank}) was applied for expression of various core peptides. In the case of production platforms a and b, lantibiotic precursor peptides are processed into their mature antimicrobially active form by existing extracellular serine protease activity. Production systems c and d enable the production of lantibiotic precursor peptides, due to the absence of the five extracellular serine proteases as a result of either the direct deletion of eight extracellular proteases or genome-reduction by 36%. PG10 (d) provides a clean secondary metabolic background due to deletion of genes involved in production of native antimicrobial compounds.

production host is the limited final cell density due to medium acidification by lactic acid that is produced during the fermentation process.[11] Although pH-controlled fermentations lead to higher cell densities, this may result in a reduced activity and stability of the lantibiotic peptide.[12] By employing *E. coli* as lantibiotic production host, higher cell densities and thus potentially higher production yields can be reached. However, heterologous expression in *E. coli* often requires additional downstream processing steps due to intracellular accumulation of the expressed peptide and the herewith associated higher chance of the formation of inclusion bodies. Another promising lantibiotic production host that possibly could overcome the previously mentioned limitations is the industrial workhorse *Bacillus subtilis*. *B. subtilis* is already extensively used for the commercial production of various proteins, but has been left relatively unexplored as potential microbial chassis for lantibiotics. In addition to having a GRAS (generally recognized as safe) status like *L. lactis*, and being able to grow to higher cell densities without medium acidification similar to *E. coli*, a great advantage of using *B. subtilis* as production host is its high natural secretion capacity.[13,14] Moreover, natural competence and efficient homologous recombination are features of *B. subtilis* that enable genetic manipulation in a relatively easy and stable manner. Furthermore, the availability of a vast extent of synthetic biology tools and optimized strains make *B. subtilis* an attractive heterologous production host.[14,15]

Several attempts have been made to employ the natural producer of subtilin *B. subtilis* ATCC 6633 or the laboratory strain *B. subtilis* 168 as a production host for lantibiotics.[16−18] For instance, nisin could be produced by *B. subtilis* ATCC 6633 after optimizing the leader peptide that was fused to the nisin core peptide.[17] In addition, a *B. subtilis* 168 strain producing antimicrobially active subtilin was constructed by integration of a 40 kb DNA region, including the subtilin gene cluster, of *B. subtilis* ATCC 6633.[18] Furthermore, Hansen et al. could diminish the sensitivity of *B. subtilis* 168 to nisin by overexpression of genes involved in the natural defense mechanism toward nisin and by introduction of the nisin immunity genes (*nisFEG* and *nisI*).[19] Although a 15-fold increase in nisin resistance could be obtained, Hanssen et al. acknowledge that the nisin level that was maximally tolerated (600 mg/L) is insufficient to enable commercial production of nisin by this engineered *B. subtilis* strain.

The degree of success of the past attempts to use *B. subtilis* as a lantibiotic production host likely has been limited by two major hurdles: (1) proteolytic processing of the lantibiotic precursor peptide by native extracellular serine protease activity of the *B. subtilis* strains that were employed, which likely caused product toxicity of the released and activated product to the host strain, and (2) the lack of a lantibiotic

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expression system that is inducible and composed of a minimal set of heterologous genes. While the benefits of *B. subtilis* as a bacterial cell factory are well-known, the previous studies did not investigate the potential of *B. subtilis* as a lantibiotic production host thoroughly. Therefore, we reasoned that it would be worthy to explore a lantibiotic production platform in *B. subtilis* in more detail. Moreover, with the development of several improved *B. subtilis* expression hosts in recent years, such as the 8-fold protease-deficient WB800 strain[20] and the genome-minimized *B. subtilis* 168 strain PG10,[21] limitations of the parental *B. subtilis* strains might be overcome.[22] Thus, in this study, we compared various *B. subtilis* strains as production hosts for lantibiotic peptides and investigated different inducible expression systems. By employing the subtilin modification and transport machinery (SpaBTC), we show that PG10 is a convenient microbial chassis for the production of various lantibiotic precursor peptides that can be activated *in vitro* at a later stage.

Our study provides a versatile platform for the screening and characterization of potent lanthipeptides that can be used in addition to previously constructed systems in *L. lactis* and *E. coli*.

RESULTS AND DISCUSSION

Production of Processed and Antimicrobially Active Lantibiotic Peptides by *B. subtilis* ATCC 6633 and *B. subtilis* 168. Although with restricted success, previous work demonstrated the production of either nisin or subtilin by *B. subtilis* ATCC 6633 or *B. subtilis* 168, respectively. However, these studies were quite limited and focused only on the production of one lantibiotic peptide. With respect to a more thorough exploration of general lantibiotic production platforms in *B. subtilis*, we re-evaluated *B. subtilis* ATCC 6633 and *B. subtilis* 168 as production hosts. *B. subtilis* 168 was chosen because it is the best-characterized and most frequently applied *B. subtilis* strain. In contrast, *B. subtilis* ATCC 6633 is not commonly used as expression host, but might provide the most optimal expression level of the different genes in the lantibiotic gene cluster, since it is the natural producer of subtilin. Furthermore, genetic engineering of *B. subtilis* ATCC 6633 is still feasible by artificial expression of the competence transcription factor ComK.[23]

To assess *B. subtilis* ATCC 6633 as a lantibiotic production host, we replaced the *spaS* gene in the subtilin gene cluster by a gene encoding a hybrid peptide composed of the subtilin leader peptide and the core peptide of either nisin (NisA) or flavucin (FlaA) (Figure 1A). Whereas nisin and its biosynthesis machinery have been thoroughly studied,[2] flavucin has recently been discovered by genome-mining and heterologous production in *L. lactis* by employing the nisin modification and transport machinery.[7] For the production of lantibiotic peptides by *B. subtilis* 168 (Figure 1B), we introduced the transcriptional unit encoding *spaBTC* in the *thrC* locus and controlled the expression by a xylose-inducible promoter. In addition, the structural lantibiotic gene was inserted in the *amyE* locus and regulated by the slightly stronger and IPTG-inducible hyperspank promoter. By using these two inducible promoters, we attempted to resemble the relative higher expression level of *spaS* compared to *spaBTC* in *B. subtilis* ATCC 6633,[24] while also circumventing the need for introduction of the *spaRK* genes in *B. subtilis* 168. As structural genes we chose the same hybrid peptides as used for *B. subtilis* ATCC 6633 as well as the native *spaS* gene, since this might provide the most optimal combination with *spaBTC*. With respect to the subtilin-nisin and subtilin-flavucin hybrid peptides in *B. subtilis* ATCC 6633 and *B. subtilis* 168, we changed the four C-terminal residues (ITPQ) of the subtilin leader peptide into the NisP cleavage site of the nisin leader peptide (ASPR) to reduce the chance of leader peptide removal by general *B. subtilis* proteases. Also, this might allow *in vitro* leader cleavage by either NisP or trypsin at a later stage.

To test the lantibiotic expression systems in *B. subtilis* ATCC 6633 and *B. subtilis* 168, mid-to-late exponentially growing cultures were induced with either supernatant (0.2%) of ATCC 6633 wild type strain (containing subtilin) or with xylose and IPTG, respectively. The presence of subtilin in the growth medium of *B. subtilis* ATCC 6633 should induce the expression of *spaS*, *spaIFEG*, and the structural gene via the two-component system SpaRK. After TCA-precipitation of the culture supernatant, antimicrobial activity was observed for *B. subtilis* ATCC 6633 and *B. subtilis* 168 expressing the flavucin core peptide as well as for the *B. subtilis* 168 strain with the native *spaS* gene, but not for the strains containing the nisin core peptide (Figure 2A,B). Possibly, modification of nisin by

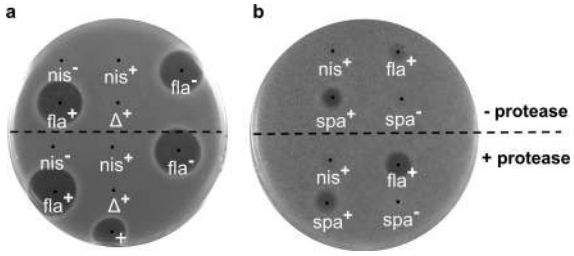

Figure 2. Antimicrobial assays to assess the production of lantibiotic peptides by*B. subtilis* ATCC 6633 and *B. subtilis* 168. TCA-precipitated supernatant was analyzed of (a) *B. subtilis* ATCC 6633 or (b) *B. subtilis* 168 strains containing *spaBTC* and various lantibiotic structural genes: subtilin-nisin hybrid peptide with ASPR as cleavage site (nis), subtilin-flavucin hybrid peptide with ASPR as cleavage site (fla), or native subtilin (spa): (+) with induction of the lantibiotic genes; (−) without induction. The black dots indicate where TCA-precipitated supernatant samples were pipetted either alone (upper half of the plate) or in combination with a protease sample (lower half of the plate); trypsin in the case of ASPR-containing peptides, *B. subtilis* 168 supernatant in the case of native subtilin. In image a, "Δ^{+}" represents the TCA-precipitated supernatant of *B. subtilis* ATCC 6633 Δ*spaS* which was used as a negative control, and "+" indicates a positive control sample for activation by trypsin.

SpaB and SpaC is less efficient compared to the subtilin and flavucin core peptides resulting in the presence of an immature subtilin-nisin precursor peptide that is more prone to degradation. This agrees with the fact that Rintala et al. could only produce nisin in *B. subtilis* ATCC 6633 after using a subtilin-nisin hybrid leader mainly composed of the nisin leader, which likely improved the modification efficiency of the nisin core peptide.[17] Strikingly, we observed that the antimicrobial activity in the culture supernatant of ATCC 6633 *spaS::spaASPR$_L$-flaA* was independent of induction with subtilin, indicating that leaky expression from the subtilin gene cluster was already sufficient for the production of antimicrobially active flavucin (possibly due to some autoinduction by flavucin). Furthermore, leader processing already occurred fully or to a large extent by *B. subtilis* protease activity for both lantibiotic expression systems in *B. subtilis* ATCC 6633 and *B. subtilis* 168. In the case of *B. subtilis* 168 containing *spaS*

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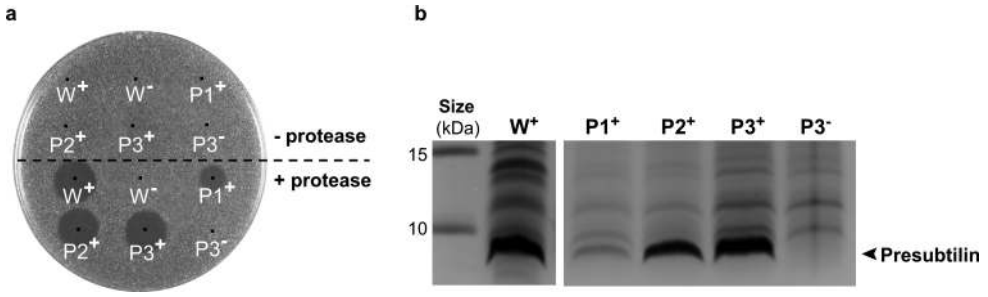

Figure 3. Production of presubtilin by *B. subtilis* WB800 and PG10. Production of presubtilin by WB800 (W) or PG10 (P1−P3) containing *spaBTC* and *spaS* under control of inducible promoters, assessed by (a) antimicrobial activity assay with or without added protease for processing and (b) tricine SDS-PAGE. TCA-precipitated supernatant was obtained from strains cultured with (+) or without induction (−) of the lantibiotic genes. P1−P3 represents three different lantibiotic expression systems in PG10: P1, *spaS* controlled by $P_{spank\text{-}hy}$ and *spaBTC* regulated by P_{xylA} by reintroduction of *xylR* in PG10; P2, *spaS* controlled by $P_{spank\text{-}hy}$ and *spaBTC* regulated by P_{spank}; P3, concerns the SURE-regulated expression system. In image a, black dots indicate where TCA-precipitated supernatant samples were pipetted either alone (upper half of the plate) or in combination with *B. subtilis* 168 supernatant for proteolytic activation of presubtilin (lower half of the plate). In image b, the expected mass of mature presubtilin is 6074 Da.

processing seemed to have occurred fully, since *in vitro* incubation with supernatant of *B. subtilis* 168 did not increase the size of the growth inhibition zone. Protease activity of both *B. subtilis* ATCC 6633 and *B. subtilis* 168 processed the subtilin-flavucin hybrid peptide to a large extent but not completely, since the growth inhibition zone slightly increased upon addition of trypsin. In agreement with this, MALDI-TOF MS indicated the presence of flavucin without the leader as well as with one to four of the C-terminal residues (ASPR) of the leader in the TCA-concentrated supernatant of *B. subtilis* ATCC 6633 *spaS::spaASPR*$_L$*-flaA* (Supplementary Figure 1).

Prevention of Leader Processing by Using *B. subtilis* WB800 or mini*Bacillus* PG10 as Lantibiotic Production Host. Although functional lantibiotic production platforms could be established in *B. subtilis* ATCC 6633 and *B. subtilis* 168, a clear disadvantage of these host strains is the occurrence of leader processing by existing and ubiquitous extracellular serine protease activity. Leader processing causes the modified lantibiotic core peptide to become antimicrobially active. Concomitantly, this hampers the application of *B. subtilis* ATCC 6633 and *B. subtilis* 168 as lantibiotic production hosts due to potential product toxicity when expressing mutant or novel lantibiotics. To circumvent the need for specific immunity genes and to achieve the production of inactive lantibiotic precursor peptides of various nature, we introduced a lantibiotic expression system in the 8-fold protease-deficient strain WB800[20] and the *B. subtilis* 168 derived mini*Bacillus* PG10 of which the genome has been reduced by 36%.[21] Both WB800 and PG10 lack all the five extracellular serine proteases of which at least AprE, WprA, and Vpr have been suggested to be involved in leader processing of presubtilin.[25] Moreover, PG10 does not secrete other antimicrobial compounds (e.g., sublancin, surfactin, and subtilosin) and thereby provides a cleaner secondary metabolic background.

First, we assessed whether WB800 and PG10 could be used for the production of the precursor peptide of subtilin, presubtilin (Figure 1C,D). For WB800, the same integration vectors were applied as used previously for 168 with the expression of *spaBTC* regulated by the xylose-inducible promoter. Since the xylose operon including the xylose repressor (XylR) had been deleted in PG10, and because PG10 is a cleaner host compared to WB800, we chose to test three different expression systems in PG10. For the first system, we reintroduced *xylR* under control of its own promoter in the *sacA* locus to reestablish the inducible expression of *spaBTC* from P_{xylA} (*thrC* locus) and to combine this with the expression of *spaS* from the hyperspank promoter (*amyE* locus), similar to the expression systems in *B. subtilis* 168 and WB800. As a second option, the expression of *spaBTC* was controlled by the P_{spank} promoter and this operon was inserted downstream of the *spaS* gene in the *amyE* locus (Figure 1D). The P_{spank} promoter is also inducible with IPTG, but is about six times less strong compared to $P_{spank\text{-}hy}$ which should again allow for a relative higher expression level of *spaS* compared to *spaBTC*. Furthermore, we tested whether the subtilin-regulated expression (SURE) system[26] could be used for the production of presubtilin. In this case, the two-component regulatory system *spaRK* with its natural promoter was inserted in the *thrC* locus, while the *amyE* locus contained *spaBTC* and *spaS* controlled by their natural promoters. The addition of subtilin to the growth medium should lead to activation of SpaRK followed by induction of gene expression of *spaBTC* and *spaS*.

As visible in Figure 3A, the TCA-precipitated culture supernatant of both WB800 and the different PG10 strains showed antimicrobial activity when mixed with supernatant of *B. subtilis* 168, indicating the presence of presubtilin in the supernatant of these strains. In agreement with this, the production of presubtilin was confirmed by tricine SDS-PAGE (Figure 3B) and MALDI-TOF MS (Supplementary Figure 2). As expected, tricine SDS-PAGE shows substantially less proteins in the culture supernatant of PG10 compared to WB800 thereby providing a cleaner production platform for lantibiotic peptides. This advantage of PG10 was also supported by the MALDI-TOF MS spectrum of WB800 by the relatively high abundance of sublancin compared to the heterologously produced presubtilin. Among the three different expression systems in PG10, the IPTG- and subtilin-controlled expression systems yielded similar amounts of presubtilin, while the system in which *spaBTC* expression is controlled by the xylose-inducible promoter showed the least production of presubtilin. For the latter system, the production of presubtilin was likely limited by lack of the AraE transporter in PG10 which normally facilitates transport of xylose in *B. subtilis*.[27] Therefore, low intracellular levels of xylose in PG10 probably caused insufficient derepression of *spaBTC*, thereby limiting modification and export of presubtilin. Nevertheless, by using the IPTG- or subtilin-controlled expression system in

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Table 1. Lantibiotic precursor peptides expressed in mini*Bacillus* PG10[a]

precursor peptide	amino acid sequence
spa_L-spaS	**MSKFDDFDLDVVKVSKQDSKITPQ**WKSESLCTPGCVTGALQTCFLQTLTCNCKISK
$spaASPR_L$-spaS	**MSKFDDFDLDVVKVSKQDSKASPR**WKSESLCTPGCVTGALQTCFLQTLTCNCKISK
spa_L-nisA	**MSKFDDFDLDVVKVSKQDSKITPQ**ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
$spaASPR_L$-nisA	**MSKFDDFDLDVVKVSKQDSKASPR**ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
spa_L-flaA	**MSKFDDFDLDVVKVSKQDSKITPQ**ITSKSLCTPGCITGWMMCNTVTKGCSFTIGK
$spaASPR_L$-flaA	**MSKFDDFDLDVVKVSKQDSKASPR**ITSKSLCTPGCITGWMMCNTVTKGCSFTIGK

[a]spa_L indicates the original subtilin leader peptide, whereas $spaASPR_L$ is the subtilin leader peptide containing the NisP cleavage site (ASPR). Leader peptide sequences are displayed in bold.

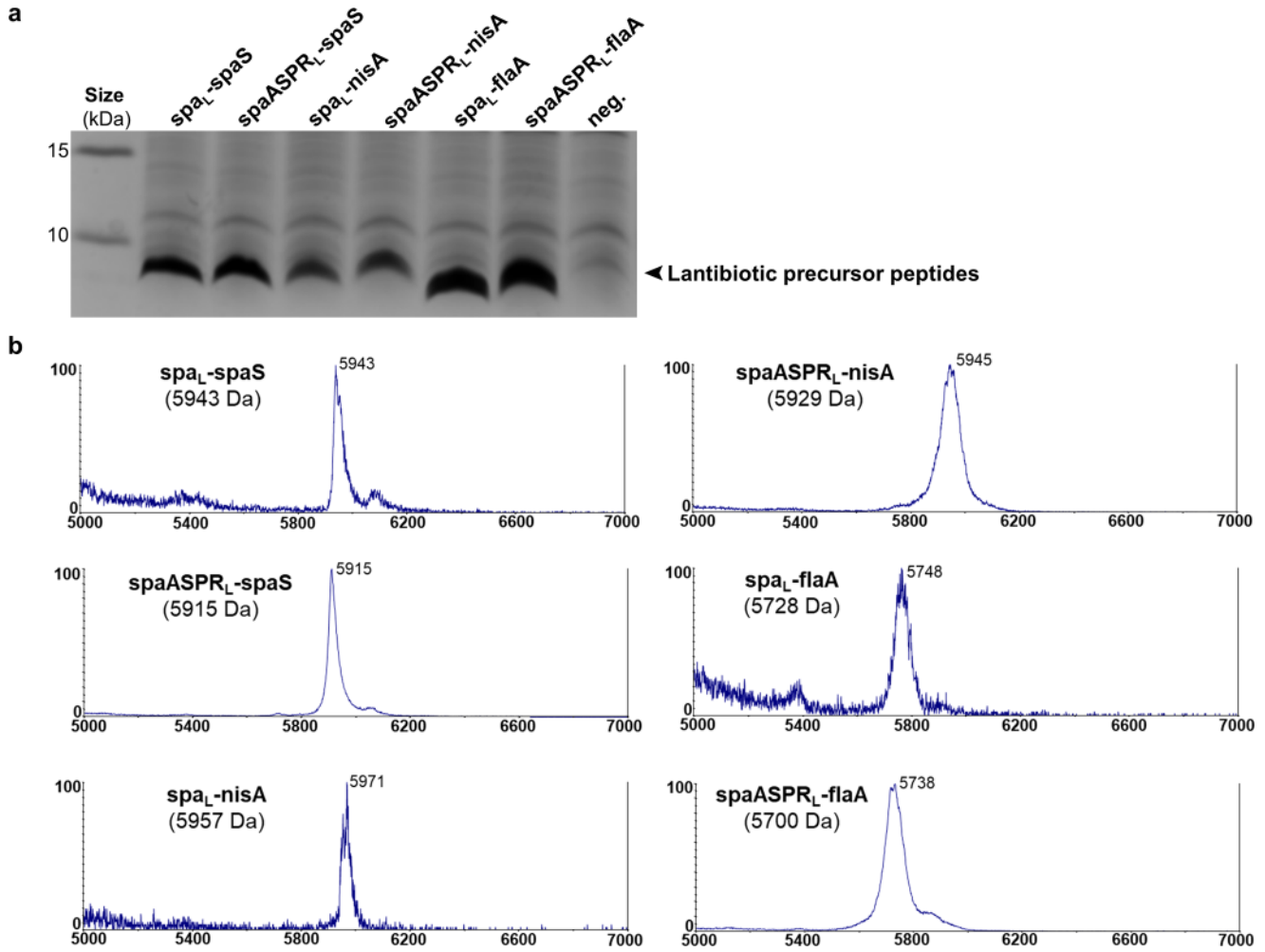

Figure 4. Production of various lantibiotic precursor peptides by *B. subtilis* PG10. Production of lantibiotic precursor peptides by PG10 strains containing P_{spank}-regulated *spaBTC* and a particular lantibiotic precursor peptide controlled by $P_{spank\text{-}hy}$ (integrated in the *amyE* locus), assessed by (a) tricine SDS-PAGE and (b) MALDI-TOF MS. The lantibiotic precursor peptide is either composed of the native subtilin leader peptide (spa_L) or the ASPR-containing subtilin leader peptide ($spaASPR_L$) and fused to the core peptide of subtilin (spaS), nisin (nisA), or flavucin (flaA). In image a, "neg." represents a negative control sample derived from PG10 containing *spaBTC* and *spaS* cultured without induction. In part b, spectra show the detected mass (in Da) of the highest peak for each of the six different precursor peptides. The theoretical mass of fully dehydrated precursor peptides without the first methionine is mentioned in parentheses.

PG10, similar production yields could be obtained as with the lantibiotic production system in WB800. Taken together, these results show that both WB800 and PG10 are suitable *B. subtilis* hosts for the production of lantibiotic precursor peptides from which the antimicrobially active core peptide later on can be released *in vitro*. An additional advantage of PG10 over WB800 is the cleaner secondary metabolic background which simplifies purification of the lantibiotic precursor peptide of interest.

Production of Various Lantibiotic Precursor Peptides by PG10. Since the production yield of presubtilin seemed comparable between WB800 and PG10, we decided to assess PG10 in more detail as a lantibiotic production host because of its cleaner secondary metabolic background. Therefore, we expressed *spaBTC* in combination with various hybrid precursor peptides composed of either the native subtilin leader peptide or ASPR-containing subtilin leader peptide fused to the core peptide of subtilin, nisin, or flavucin (Table

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Table 2. Processing of Various Lantibiotic Precursor Peptides Produced by mini*Bacillus* PG10[a]

	incubated with								
Precursor peptide	168*	ATCC 6633Δ*spaS**	NisP	Trypsin	AprE*	WprA#	Vpr*	Bpr*	Epr*
spa_L-spaS	+	+	−	−	+	+	−	+	−
$spaASPR_L$-spaS	±	−	+	+	±	−	−	+	−
spa_L-nisA	±	−	−	−	−	−	−	−	−
$spaASPR_L$-nisA	−	−	+	+	−	−	−	−	−
spa_L-flaA	−	−	−	−	−	−	−	−	−
$spaASPR_L$-flaA	−	−	−	±	−	−	−	−	−

[a]The agar diffusion test was performed with TCA-precipitated supernatant of PG10 strains producing various lantibiotic precursor peptides. TCA-precipitated supernatant was mixed with culture supernatant (depicted as *) of *B. subtilis* 168, *B. subtilis* ATCC 6633 Δ*spaS*, or PG10 protease overexpression strains, NisP, trypsin, or cell lysate of PG10 overexpressing WprA (depicted as #). Processing abilities of the different protease samples were assessed by observation of growth inhibition zones, which were divided in three categories: no growth inhibition zone (diameter <1 mm; indicated by −), growth inhibition zone with a diameter between 1 and 10 mm (indicated by ±), and growth inhibition zone with a diameter >10 mm (indicated by +). See Supplementary Table 1 for exact diameters of growth inhibition zones.

1). Production and secretion of all the various precursor peptides into the growth medium of PG10 was observed, including the precursor peptides containing the nisin core peptide (Figure 4A). Herewith the advantage of a lantibiotic production system in a *B. subtilis* strain that lacks extracellular serine protease activity is clearly demonstrated since even peptides can be produced that could not be obtained by using *B. subtilis* ATCC 6633 or *B. subtilis* 168 as production host. Along with a greater variety of peptides, higher production yields can be obtained with the production of lantibiotic precursor peptides. Furthermore, MALDI-TOF MS showed that the detected masses of the various precursor peptides correlated to the theoretical masses of fully or almost fully modified precursor peptides (Figure 4B). This indicates that SpaB and SpaC display a broad substrate specificity, resulting in a high degree of modification of the various precursor peptides.

Next, we assessed whether the various precursor peptides could be processed by protease activity of either a supernatant of a *B. subtilis* 168 or a *B. subtilis* ATCC 6633 Δ*spaS* overnight culture, NisP, or trypsin (Table 2 and Supplementary Table 1). Whereas supernatant of *B. subtilis* 168 could release antimicrobially active subtilin from both types of leader peptides as well as nisin from the native subtilin leader peptide, it could not activate the precursor peptides containing flavucin as core peptide. Strikingly, the proteolytic activity in the supernatant of the *B. subtilis* ATCC 6633 Δ*spaS* overnight culture could only release antimicrobially active subtilin from the native subtilin leader peptide. These observations agree with previous speculations[28,29] indicating that the substrate specificity of extracellular serine proteases of *B. subtilis* is affected by the N-terminal residues of the core peptide, in addition to the residues that constitute and precede the cleavage site. In agreement with this, the first two N-terminal residues of subtilin are a tryptophan and lysine, while both nisin and flavucin core peptides start with isoleucine followed by threonine. Possibly, the N-terminal tryptophan of the subtilin core peptide plays an important role in presubtilin processing by *B. subtilis* extracellular proteases. In addition, our data suggest that extracellular serine proteases of *B. subtilis* ATCC 6633 rely more on the native cleavage site in presubtilin for release of the subtilin core peptide compared to extracellular serine proteases of *B. subtilis* 168. However, the *in vitro* conditions used in this experiment might not have been optimal for the extracellular serine proteases of both *B. subtilis* 168 and *B. subtilis* ATCC 6633 in order to process the various precursor peptides. By varying pH and salt concentration a broader substrate tolerance might be observed, since these factors affect the substrate specificity of proteases.[29]

In addition to the *in vitro* release of mature subtilin by proteolytic activity of *B. subtilis* supernatant, we could release each of the three core peptides from the ASPR-containing subtilin leader peptide by using NisP or trypsin. While antimicrobially active subtilin or nisin could be obtained by using either NisP or trypsin, flavucin could only be released by treatment with trypsin. The inability of NisP to release flavucin might be related to the slight positive charge on the N-terminal part of the flavucin core peptide compared to the rather neutral N-terminal part of the subtilin and nisin core peptides. Again, different reaction conditions might allow the *in vitro* release of flavucin from the ASPR-containing subtilin leader peptide by NisP.

Overexpression of *B. subtilis* Extracellular Serine Proteases as *in Vitro* Leader Processing Tool. The successful heterologous production of presubtilin and other precursor peptides in PG10 allowed us to gain more insight in the proteolytic abilities of the five extracellular serine proteases (AprE, WprA, Vpr, Bpr, Epr) of *B. subtilis*. Also, this might provide a neater way for the *in vitro* processing of presubtilin and allow application of these proteases for *in vitro* processing of other precursor peptides. Therefore, we overexpressed each of the proteases, using the coding sequences of *B. subtilis* ATCC 6633, in PG10. After induction of protease expression in PG10, we analyzed whether protease activity in the culture supernatant could activate the various precursor peptides produced by PG10. Although WprA was previously detected in the culture supernatant of *B. subtilis* 168,[29] we also analyzed the cell lysate of PG10 overexpressing WprA, since it is a cell wall-associated protease.[30] Our results show that the supernatant of PG10 overexpressing AprE and Bpr as well as the cell lysate of PG10 WprA could activate presubtilin and presubtilin containing the NisP cleavage site (Table 2). In contrast, Epr- and Vpr-containing supernatant could not process any of the precursor peptides, including the native subtilin precursor peptide.

Hereby, we provide for the first-time direct evidence for a prominent role of AprE, WprA, and Bpr in leader peptide cleavage of presubtilin. Although the proteolytic ability of AprE and WprA to process presubtilin agrees with the study of Corvey et al.,[25] we did not find a role for Vpr in presubtilin processing in comparison to the findings of Corvey et al. Rather, our study reveals Bpr as the third extracellular serine

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protease capable of activating presubtilin. Although we provided direct evidence for the proteolytic ability of AprE, WprA, and Bpr to cleave presubtilin, we cannot rule out a role for Vpr and Epr in presubtilin processing since factors for maturation into their active forms might have been lacking in PG10. With these PG10 strains at hand, future experiments could provide even more insight in the substrate specificities of the five extracellular serine proteases of *B. subtilis* and assess whether they can be used for the *in vitro* activation of other lantibiotic peptides.

CONCLUSION

Although various lantibiotic peptides have been successfully identified via their heterologous production in *E. coli* or *L. lactis*, two beneficial traits of these bacterial hosts can be combined by employing *B. subtilis* as an expression host: high cell density cultures and direct secretion of lantibiotic peptides into the culture medium. Our study demonstrates that these benefits indeed can be met and that *B. subtilis* can serve as an excellent lantibiotic production host by choosing a *B. subtilis* strain that lacks extracellular serine protease activity. In this way, leader processing during expression of the lantibiotic genes is prevented, thereby circumventing the major obstacle of *B. subtilis* as a production host for lantibiotics. The NisP cleavage site can be used conveniently in most cases, but if inefficient also a factor Xa site can be engineered instead, or an extracellular serine protease of *B. subtilis* can be attempted. By employing WB800 or mini*Bacillus* PG10, lantibiotic precursor peptides can efficiently be produced and secreted. The production of lantibiotic precursor peptides prevents toxicity to the production host and alleviates the need of immunity genes. Furthermore, PG10 provides as additional advantage a clean secondary metabolic background which might simplify downstream processing and characterization of the produced lantibiotic precursor peptide.

Thus, by using the subtilin modification and transport enzymes the established lantibiotic production platform in mini*Bacillus* PG10 allows a convenient way to characterize potent lantibiotic peptides. Furthermore, this opens possibilities for investigation of *B. subtilis* as an expression host for other classes of antimicrobial peptides, thereby accelerating the identification of novel natural products that potentially can be applied to combat antimicrobial resistance.

METHODS

Strains and Plasmids. Bacterial strains and plasmids used in this study are listed in Supplementary Table 2. *B. subtilis* strains were cultured in LB-Lennox medium (Formedium) at 37 °C with aeration. *E. coli* (MC1061, DH5α or Top10) used as cloning host and *M. luteus* used as indicator strain were grown in LB at 37 °C with aeration. When required, antibiotics were added to the growth media of *B. subtilis* at the following concentrations: 100 μg/mL spectinomycin, 0.5 μg/mL erythromycin, 12.5 μg/mL lincomycin, or 5 μg/mL kanamycin. Half of the concentration of antibiotics was used in the case of liquid cultures of PG10. For *E. coli*, 100 μg/mL ampicillin or 100 μg/mL kanamycin was used. Growth media was supplemented with agar (1.5%) for solid medium.

Construction of Plasmids. Conventional cloning techniques were applied for construction of all plasmids. *spa* genes and extracellular serine protease genes were taken from *B. subtilis* ATCC 6633 (NCBI Reference Sequence: NZ_ADGS00000000.1). Oligonucleotides are listed in Supplementary Table 3 and were purchased from Biolegio. Restriction enzymes, T4 DNA ligase, and Phusion-HF DNA polymerase were obtained from Thermo Fisher Scientific. PfuX7 DNA polymerase (purified in our lab) was used for cloning purposes and colony PCR. Unless otherwise mentioned, standard restriction−ligation was used for construction of plasmids. Quick-Fusion ligation was performed according to manufacturer's descriptions (Bimake.com). All constructs were verified by sequencing. *B. subtilis* 168, WB800, and pGSP12-containing *B. subtilis* ATCC 6633 were transformed in Spizizen's minimal medium as described before,[31] except that 2.5 μg/mL erythromycin was added to the culture medium in the case of ATCC 6633. For transformation of *B. subtilis* 168 PG10 carrying the competence genes (*comK, comS*) under the control of the mannitol-inducible promoter (P_{mtlA}),[32] competence was induced by adding 0.5% (w/v) mannitol.

Lantibiotic Gene Expression Constructs. To construct pDG1664-P_{xylA}-spaBTC the *spaBTC* genetic sequence was obtained from the genomic DNA of *B. subtilis* ATCC 6633 by PCR amplification using primer pairs ASRo254 + ASRo255, followed by *NotI* and *NheI* digestion and T4 ligation into similarly digested pDG1664.

To construct pDR111-$P_{spank\text{-}hy}$-spaS, *spaS* was amplified from the genomic DNA of *B. subtilis* ATCC 6633 by using primer pair ASRo256 + ASRo257. The *spaS* PCR product was digested with *Hin*dIII and *NheI* and ligated into *Hin*dIII-*NheI*-digested pDR111 yielding pDR111-$P_{spank\text{-}hy}$-*spaS*. Overlap extension PCR was used in order to obtain the pDR111 constructs with different types of lantibiotic core peptides fused to the native subtilin leader sequence or the subtilin leader sequence containing the NisP cleavage site (ASPR). Primer pairs ASRo298 + ASRo241, ASRo290 + ASRo291, and ASRo294 + ASRo291 were used to amplify the *spaS*, *nisA*, and *flaA* core peptide sequences from pDR111-$P_{spank\text{-}hy}$-spaS, pNZE3-nisA, and pNZE3-flaA, respectively. To obtain the ASPR-modified *spaS* leader sequence for the *spaS* core peptide constructs, primer pair ASRo224 + ASRo297 were used. Primer pairs ASRo224 + ASRo289 and ASRo224 + ASRo288 were used to amplify the native *spaS* leader sequence or the *spaS* leader sequence containing the ASPR cleavage site respectively for the *nisA* core peptide constructs. To obtain the analogous constructs for the *flaA* core peptide sequence, primer pairs ASRo224 + ASRo293 and ASRo224 + ASRo292 were used. In all cases, pDR111-$P_{spank\text{-}hy}$-spaS was used as template DNA for amplification of the subtilin leader peptide sequences. To create PCR fusion products of a particular leader and core peptide sequence, the PCR products of the particular leader and core peptide were mixed together in a PCR reaction and a primer pair was added allowing for amplification of the fusion product. To generate *spaASPR$_L$-spaS* primers, ASRo224 and ASRo241 were used. To obtain *spa$_L$-nisA*, *spaASPR$_L$-nisA*, *spa$_L$-flaA*, and *spaASPR$_L$-flaA* primers ASRo224 and ASRo291 were used. The PCR fusion products were digested with *Eco*RI and *SphI* and ligated into similarly digested pDR111 yielding the pDR111 vectors containing $P_{spank\text{-}hy}$ and the various structural genes. To insert P_{spank}-*spaBTC* into the various pDR111 vectors, pDR111-$P_{spank\text{-}hy}$-spaS and pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-spaS were linearized by PCR via primer pair 333-pDR-Eco_fw and 334-pDR-Eco_rv, whereas the four pDR111 vectors containing either *nisA* or *flaA* were linearized using primer pair 375-pDR-Phs-

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bb-fw-Eco and 376-pDR-Phs-bb-rv-Eco. The P_{spank}-*spaBTC* insert was amplified from pDG1664-P_{spank}-spaBTC using primer pair 291_fw_Pspank and 335-pDG-Ps-BTC-Eco-rv. *BsaI*-digestion of the pDR111 precursor backbones and P_{spank}-*spaBTC* yielded the pDR111 vectors containing P_{spank}-*spaBTC* downstream of one of the six different precursor peptides. The plasmids obtained were named pDR111-$P_{spank\text{-}hy}$-spaS-P_{spank}-spaBTC, pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-spaS-P_{spank}-spaBTC, pDR111-$P_{spank\text{-}hy}$-spa$_L$-nisA-P_{spank}-spaBTC, pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-nisA-P_{spank}-spaBTC, pDR111-$P_{spank\text{-}hy}$-spa$_L$-flaA-P_{spank}-spaBTC, and pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-flaA-P_{spank}-spaBTC.

To construct pDR111-P_{spaS}-spaBTC-P_{spaS}-spaS, pDR111 was linearized and $P_{spank\text{-}hy}$ was removed by PCR amplification with primers 145-pDR111-QF-F and 146-pDR111-QF-R. The *spaBTC* and *spaS* coding regions including their native promoters were PCR amplified from the genomic DNA of *B. subtilis* ATCC 6633 by using primer pairs 147-spaBTCS-F and 148-spaBTCS-R. Quick-Fusion ligation resulted in pDR111-P_{spaS}-spaBTC-P_{spaS}-spaS.

To construct the four different pJOE8999 vectors for deletion of *spaS*, replacement of *spaS* by *spaASPR$_L$-nisA* or *spaASPR$_L$-flaA* (in *B. subtilis* ATCC 6633), or for reintroduction of *xylR* in *sacA* (in PG10), a specific single guide RNA (sgRNA)-encoding sequence (Supplementary Table 3) was designed by using the CRISPR Guide Design Software of Benchling and cloned into pJOE8999 via *BsaI* digestion. To allow homologous recombination, up- and downstream flanking regions to construct pJOE_ΔspaS were obtained from the genomic DNA of *B. subtilis* ATCC 6633 using primer pairs ΔspaS_up-fw + ΔspaS_up-rv and ΔspaS_down-fw + ΔspaS_down-rv. *BsaI*-digestion of PCR products followed by ligation into the similarly digested pJOE vector containing the *spaS* spacer sequence yielded pJOE_ΔspaS. To construct pJOE_nisA and pJOE_flaA, flanking regions were obtained from the genomic DNA of *B. subtilis* ATCC 6633 via primer pairs 209-spaS_up-F-SfiI + 210-spaS_up-R-SfiI and 211-spaS_down-F-SfiI + 212-spaS_down-R-SfiI, respectively. The *spaASPR$_L$-nisA* and *spaASPR$_L$-flaA* inserts were amplified from pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-nisA and pDR111-$P_{spank\text{-}hy}$-spaASPR$_L$-flaA by using 206-spaL-F-SfiI as forward primer and 207-nisA-R-SfiI or 208-flaA-R-SfiI as reverse primer, respectively. To obtain the final vectors of pJOE_nisA and pJOE_flaA, flanking regions and inserts were digested with *SfiI* followed by ligation into the similarly digested pJOE vector with the *spaS* spacer sequence. To construct pJOE_xylR, primer pairs 275-sacA1-Fw-Eco + 276-sacA1-Rv-Eco and 279-sacA2-Fw-Eco + 280-sacA2-Rv-Eco were used for amplification of the flanking regions, while *xylR* with its natural promoter was amplified from the genomic DNA of *B. subtilis* 168 using primers 277-XylR-Fw-Eco + 278-XylR-Rv-Eco. After linearization of pJOE with the spacer sequence for recognition of *sacA* via PCR with primers 281-pJOE_sacA-Eco and 282-pJOE_sacA-Eco, *BsaI*-digestion of the PCR products followed by ligation yielded the final pJOE_xylR vector.

Protease Gene Expression Constructs. The genetic sequences of *wprA*, *aprE*, *vpr*, *epr*, and *bpr* were PCR amplified from the genomic DNA of *B. subtilis* ATCC 6633 by using the following primer pairs: 77-wprA-F-*Sal*I + 79-wprA-R-PaeI, 81-aprE-F-*Sal*I + 83-aprE-R-PaeI, 86-vpr-F-NheI + 87-vpr-R-PaeI, 105-epr-F-*Sal*I + 106-epr-R-NheI, and 108-bpr-F-*Sal*I-RBS + 109-bpr-R-NheI, respectively. PCR products were digested according to the restriction sites introduced by PCR amplification and ligated into similarly digested pDR111, yielding the various pDR111 protease overexpression vectors.

Production of Lantibiotic Peptides and Proteases. For the heterologous expression of the lantibiotic or protease genes by various *B. subtilis* strains, overnight cultures were diluted to a final OD_{600} of 0.075 into 50 mL of fresh LB and grown at 37 °C and 220 rpm. Cultures were induced at midexponential phase with either 0.2% (v/v) of culture supernatant from *B. subtilis* ATCC 6633 (containing subtilin), 1% xylose, and/or 1 mM IPTG. After 3 h, the growth medium was separated from the cells by centrifugation, and in case of the lantibiotic gene expression strains the culture supernatant was concentrated (125-fold) by trichloroacetic acid (TCA) precipitation. For this purpose, peptide pellets were obtained by centrifugation (1 h, 9000 rpm, 4 °C), washed with ice-cold acetone (1 h, 8500 rpm, 4 °C), air-dried, and solubilized in 0.05% acetic acid. The cell lysate fraction of PG10 WprA was obtained by bead beating of cells resuspended in 30 mM Tris-HCl pH 7.5 containing 0.5% *n*-dodecyl *β*-maltoside (DDM).

Peptide Detection. For tricine SDS-PAGE, a 16% separating gel was used. After separation of proteins, proteins were visualized by using Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, United States).

For MALDI-TOF MS, a 1 μL sample of TCA-precipitated culture supernatant was spotted and dried on the target. Subsequently, 1 μL of matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid) was spotted on top of the sample. Mass spectra were collected by using a Voyager DE PRO matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems). For analysis of the mass spectra "Data Explorer" software version 4.0.0.0 (Applied Biosystems) was used.

Antimicrobial Activity Assay. To assess antimicrobial activity, indicator plates were prepared composed of LB agar and 0.2% (v/v) of an overnight culture of *M. luteus*. An 8 μL aliquot of the TCA-precipitated culture supernatant was pipetted on the plate, and if desired, combined with 2 μL of purified NisP or trypsin (0.1 mg/mL), 4 μL of *B. subtilis* 168 supernatant, or 4 μL of supernatant or cell lysate derived from the PG10 protease overexpression strains. For Table 2, diameters of growth inhibition zones were measured to assess the ability of various protease-containing samples to activate lantibiotic precursor peptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00194.

MALDI-TOF MS analysis of TCA-precipitated supernatant of ATCC 6633 *spaS::spaASPR$_L$-flaA*.; production of presubtilin by WB800 and PG10; diameters of growth inhibition zones after incubation of PG10-derived lantibiotic precursor peptides with various protease-containing samples; lists of plasmids, bacterial strains, and oligonucleotides used in this study (PDF)

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A.Y.v.T., A.J.v.H., N.A.W.d.K., and A.S.R. designed, performed, and analyzed experiments. A.Y.v.T., A.J.v.H., and O.P.K. wrote the manuscript. A.Y.v.T. prepared the figures. J.S. provided the genome-reduced *B. subtilis* 168 PG10 strain and gave advice about its properties and use. O.P.K. conceived the project and directed the research.

Notes

The authors declare no competing financial interest.

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