Autoregulation of subtilin biosynthesis in Bacillus subtilis: the role of the spa-box in subtilin-responsive promoters

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Abstract
The production of the type I antimicrobial peptide (AMP) subtilin by Bacillus subtilis is regulated in a cell-density-dependent manner [Kleerebezem M, de Vos WM, Kuipers OP. The lantibiotics nisin and subtilin act as extracellular regulators of their own biosynthesis. In: Dunny GM, Winans SC, editors. Cell–cell signaling in bacteria. Washington, D.C., USA: ASM Press; 1999. p. 159–74; Stein T, Borchert S, Kiesau P, Heinzmann S, Kloss S, Klein C, Helfrich M, Entian KD. Dual control of subtilin biosynthesis and immunity in Bacillus subtilis. Mol Microbiol 2002;44:403–16; Stein T, Heinzmann S, Kiesau P, Himmel B, Entian KD. The spa-box for transcriptional activation of subtilin biosynthesis and immunity in Bacillus subtilis. Mol Microbiol 2003;47:1627–36]. Three subtilin-responsive promoter elements within the spaBTCSIFEGRK are controlled by the specific cis-acting sequence element called the spa-box, which represents the binding site of the subtilin regulator SpaR [Stein T, Heinzmann S, Kiesau P, Himmel B, Entian KD. The spa-box for transcriptional activation of subtilin biosynthesis and immunity in Bacillus subtilis. Mol Microbiol 2003;47:1627–36]. Here, we describe the functional characterization of the spaB, spaS and spaI promoters by transcriptional fusion with a promoterless gusA gene. Within these gusA fusion constructs, transcription initiation start sites of the spaS and spaI promoters were mapped to be located downstream of the spa-box, which is in contrast to previous reports [Banerjee S, Hansen JN. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J Biol Chem 1988;263:9508–14; Stein T, Heinzmann S, Kiesau P, Himmel B, Entian KD. The spa-box for transcriptional activation of subtilin biosynthesis and immunity in Bacillus subtilis. Mol Microbiol 2003;47:1627–36]. Nevertheless, all spa-promoters displayed typical cell-density-dependent activity in a subtilin-producing strain B. subtilis ATCC6633. Moreover, analysis of β-glucuronidase activities in a spaB mutant of B. subtilis ATCC6633 and a derivative of strain 168 that harbors the spaRK genes integrated in the chromosomal amyE locus, confirmed that these promoters are activated by subtilin-triggered, SpaRK-mediated, quorum-sensing control. Quantitative analysis showed that the spaS promoter strength at a given subtilin concentration appeared to be approximately five-fold higher than the spaB promoter, which in turn is approximately two-fold higher than the spaI promoter. Finally, it is shown that the elementary components involved in subtilin-mediated regulation are the two-component system, SpaRK, and a spa-box containing promoter.

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Keywords: Bacillus subtilis; Subtilin; Peptide pheromone; Regulation; Quorum sensing

1. Introduction

Subtilin is a type I antimicrobial peptide (AMP) or lantibiotic that is produced by Bacillus subtilis. Lantibiotics are peptide-derived antibiotics with high antimicrobial activity against various Gram-positive bacteria, including pathogenic bacteria such as propionibacteria, staphylococci, clostridia,
enterococci and streptococci. This family of AMPs is characterized by the presence of unusual amino acids like dehydroalanine, dehydrobutyryne and the typical lantionine or β-methyl-lantionine bridges.

Biosynthesis of subtilin by *B. subtilis* is dependent on the products of at least 10 genes, i.e. spaBTCSEFEGRK that are organized in a gene cluster (for a review, see [30]). The spaS gene encodes the ribosomally synthesized subtilin precursor in which the amino acid modifications are introduced enzymatically by the products of spaB and spaC. The modified precursor is then transported across the cytoplasmic membrane by the ABC transporter encoded by spaT, which has been shown to form a membrane-associated complex with the modification enzymes SpaB and SpaC ([14,21]). Follow-

ing transport, the N-terminal leader peptide of the modified subtilin precursor is removed by the activity of unspecific proteases secreted by *B. subtilis* to release the mature and active AMP ([7]). The producing cell is protected against the antimicrobial activity of subtilin by a producer-immunity system composed of SpaL, SpaE, SpaF and SpaG ([19]). Finally, the gene products of spaR and spaK share sequence homology with the response regulator and sensor proteins of bacterial two-component regulatory systems, respectively, and are involved in subtilin regulation ([20]). Subtilin production is regulated in a growth-phase-dependent manner, starting in exponential growth phase and increasing to reach maximal level at the beginning of the stationary phase ([13,16,31,32]). Production of subtilin is regulated at the transcriptional level ([2,31,32]). Three promoters have been identified upstream of spaB, spaS and spaC, which drive the regulation of the transcriptional units encompassing spaBCT, spaS and spaTFEG, respectively ([16,31,32]). Control of these promoters is mediated by the intercellular signal-sensing control module that has also been described for the production of the structurally homologous lantibiotic nisin that is produced by *Lactococcus lactis* ([11,18,22]). This mode of control involves the peptide pheromone activity of subtilin itself that interacts with the subtilin-specific sensor kinase SpaK, leading to SpaR activation and subsequent binding to the subtilin-responsive promoter elements ([16,31,32]). Recently, it has been shown that SpaR binds to a specific target sequence within the subtilin-responsive promoter elements, designated the spa-box, which is a pentanucleotide direct repeat (PDR) sequence separated by six nucleotides ([32]). However, the location of this spa-box relative to the transcription start site appears to be variable in the different promoters, placing this sequence element either upstream of the transcription start site (in the spaB promoter), or downstream of the transcription start site (in the spaS and spaC promoters) ([2,31,32]).

Moreover, two different transcription start sites of the spaS promoter have been reported, located 74 and 223 base pairs upstream of the spaS start codon ([2,31,32]). Next to the three subtilin-responsive promoters, a fourth promoter upstream of spaR, and driving expression of the spaRK genes, appeared to be regulated by the transition state regulatory system that involves the abrB and sigH gene products, thereby, placing subtilin production under dual cell-density-dependent control ([31]).

Here, we describe the construction of transcriptional fusions between the spaB, spaS and spaC promoters, and a promoterless copy of the β-glucuronidase-encoding *Escherichia coli* gusA gene. In these constructs, the spaS and spaC promoter transcription start sites were mapped closer to their corresponding genes as compared to previous reports ([2,31]). Nevertheless, these spa promoter gusA constructs displayed typical subtilin-mediated regulation of activity, including cell-density-dependent regulation in a subtilin producer. SpaRK-dependent activation following induction with extracellular addition of subtilin. Finally, introduction of the spa-box-specific pentanucleotides in the structurally homologous nisA promoter ([8]) resulted in a subtilin-responsive ‘hy-

2. Materials and methods

2.1. Bacterial strains and growth conditions

*B. subtilis* strain ATCC6633 (subtilin producer), its spaB derivative ([21]), strain 168 and its amyE::spaRK derivative (NZ8900) were grown in two-fold concentrated L-broth medium (2*LB) ([29]) with aeration at 37 °C. *E. coli* MC1061, which was used as an intermediate host for cloning, was grown in L-broth ([29]) based media with aeration at 37 °C. Strain NZ8900 was constructed by single cross-over homologous recombination of pNZ8900 in the *amyE* locus of the *B. subtilis* 168 chromosome and selection of kanamycin-resistant integrants. Correct integration of pNZ8900 was confirmed by PCR analysis. The antibiotics chloramphenicol (10 or 5 μg/ml) for plasmid encoded and chromosomally encoded resistance marker, respectively), kanamycin (10μg/ml) and tetracyclin (10 ng/ml) were added when appropriate.

2.2. Plasmids and DNA manipulations

Plasmids used in this study and their relevant characteristics are listed in Table 1. Plasmid DNA was isolated as described previously ([3]), followed by CsCl/ethidium bromide isopycnic centrifugation. Recombinant DNA techniques were performed essentially as described ([29]). Restriction endonucleases, T4-DNA ligase and *Klenow* fragment of *E. coli* DNA polymerase were used as recommended by the manufacturers (Amersham-Pharmacia).

Promoter fragments derived from the subtilin gene cluster were amplified by PCR using *Pwo* DNA-polymerase (Boehringer, Manheim, Germany) according to the manufacturer’s protocol. Plasmid DNA (10 ng) or chromosomal DNA from *B. subtilis* ATCC6633 (100 ng) was used as DNA template, and the primer-couples used (100 ng each) and their location within the subtilin gene cluster are listed in Table 2. Three fragments of 142 bp (primer-combination BF and
Finally, the mutant above; the resulting plasmid was designated pNZ8091-2.

Table 1

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<tr>
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<sup>a</sup> Cam<sup>b</sup>, chloramphenicol resistant; Tef<sup>b</sup>, tetracyclin resistant.

<sup>b</sup> A generous gift of Dr. S. Bron of the Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands.

Table 2

| Primer sequences and their location in the subtilin gene cluster are indicated by the number of the base directly on the 3'-side of the restriction site sequence (numbering of base pairs is based on complete subtilin gene cluster coding sequence, accession number BSU09819, total length: 14616 base pairs). Primer sequences and their location in the subtilin gene cluster are indicated by the number of the base directly on the 3'-side of the restriction site sequence (numbering of base pairs is based on complete subtilin gene cluster coding sequence, accession number BSU09819, total length: 14616 base pairs). Primer sequences and their location in the subtilin gene cluster are indicated by the number of the base directly on the 3'-side of the restriction site sequence (numbering of base pairs is based on complete subtilin gene cluster coding sequence, accession number BSU09819, total length: 14616 base pairs). Primer sequences and their location in the subtilin gene cluster are indicated by the number of the base directly on the 3'-side of the restriction site sequence (numbering of base pairs is based on complete subtilin gene cluster coding sequence, accession number BSU09819, total length: 14616 base pairs).

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<tr>
<td>SPARF</td>
<td>GCCAACTGGTTAAAAGAATTTATC</td>
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<sup>a</sup> Cam<sup>b</sup>, chloramphenicol resistant; Tef<sup>b</sup>, tetracyclin resistant.

<sup>b</sup> A generous gift of Dr. S. Bron of the Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands.

<sup>c</sup> Spa<sup>c</sup> promoter derivative, pNZ8089, was removed by Bgl<sup>II</sup> digestion and replaced by the tetracyclin resistance marker isolated as a BamHI–HindIII fragment from pNZ280 (30), after filling in the cohesive ends using the Klenow fragment.
of DNA polymerase I of *E. coli*. In the resulting plasmids the orientation of the *xer* gene was determined by *Bam*HI-*EcoRI* digestion, and those clones that contained the *xer* gene in opposite orientation relative to the *gusA* gene were used in further experiments. The resulting plasmids were designated pNZ273Tet, pNZ8092 (*spaS* promoter), pNZ8093 (*spaB* promoter), pNZ8094 (*spaI* promoter), pNZ8095 (*spaR* promoter) and pNZ8096 (*hybrid* *spanis* promoter) (Table 1).

The *spaRR* integration plasmid pNZ8900 was constructed by PCR amplification of the *spaRR* locus, including the *spaR* promoter region, using the primers PSPARF and SPARKR (Table 2) and chromosomal DNA of *B. subtilis* ATCC6633 as a template. The resulting PCR product was digested with *Bam*HI and *Xba*I, and cloned into similarly digested pBT2K (a generous gift of Dr. S. Bron of the Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands). The resulting plasmid was used for single cross-over integration into the *B. subtilis* 168 chromosome (see above).

DNA sequences of the cloned promoter fragments were verified by automatic DNA sequence analysis with an ALF DNA sequencer (Pharmacia Biotech). Sequence reactions were performed according to the manufacturer’s protocols, using the autorad sequencing kit and a fluorescein-labeled GUSR primer (Table 2).

2.3. Transcription start site determination

*Spa*-promoter-derived transcription start positions were determined by primer extension, which was performed by annealing 20 ng of the GUSR primer to 100 μg of RNA as was previously described [23]. RNA was isolated from *B. subtilis* NZ8000 (168RK) harboring pNZ8089, pNZ8090, pNZ8091 or pNZ8091-2, grown to an optical density at 600 nm (OD600) of 1.0 and subsequently induced with variable levels of subtilin for 24 nucleotides relative to the transcription start site [8,16,17], which is identical to that found for the *spa*-box in subtilin-mediated transcription initiation. Moreover, the pentanucleotide direct repeats identified within the nisin-responsive promoters (upstream of the *nisA* and *nisF* genes) of the homologous system found in *L. lactis* are both located at –39 to –24 nucleotides relative to the transcription start site [8,16,17], which is identical to that found for the *spa*-box within the *spaR* promoter [31,32].

In our laboratory, we have constructed several putative *spa* promoter fusions with the promoterless *gusA* gene in the promoter probe vector pNZ273 [28], which either lack or contain the previously described *spaS* pro-

2.5. β-glucuronidase assays

Qualitative β-glucuronidase activity screening assays were performed on plates containing 0.5 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu; Research Organics Inc., Cleveland, Ohio).

Quantitative β-glucuronidase activity measurements were performed using a procedure adapted from de Ruyter et al. [8]. To measure β-glucuronidase activity in subtilin-producing cells, cells were harvested by centrifugation and resuspended in phosphate buffer (0.1 M 

2.4. Transformation of *B. subtilis* strains

*B. subtilis* strains were transformed by electroporation. Cells were grown in 2-LB to an optical density at 600 nm (OD600) of 1.0–1.5, and harvested immediately after electroporation, 1.9 ml of 2-LB was added and cells were incubated 60–90 min under aerobic conditions to allow expression of resistance markers. Finally, 10–100 μl of cells was plated on selective 2-LB plates. In general, transformation efficiencies of 10^3–10^4 colony forming units per μl of plasmid DNA were obtained.

3. Results

3.1. The *spa*-promoters

The variation of the location of the *spa*-box relative to the transcription start site in the subtilin-responsive promoter elements [2,31,32] is an intriguing finding. In addition, the discrepancy in the reported transcription start sites of the *spaS* promoter [2,31] is confusing with regard to the exact role of the *spa*-box in subtilin-mediated transcription initiation. Moreover, the pentanucleotide direct repeats identified within the nisin-responsive promoters (upstream of the *nisA* and *nisF* genes) of the homologous system found in *L. lactis* are both located at –39 to –24 nucleotides relative to the transcription start site [8,16,17], which is identical to that found for the *spa*-box within the *spaR* promoter [31,32].

In our laboratory, we have constructed several putative *spa* promoter fusions with the promoterless *gusA* gene in the promoter probe vector pNZ273 [28], which either lack or contain the previously described *spaS* pro-
The spa-promoters. Panel A: Schematic representation of the subtilin biosynthesis gene cluster (spaBTCSIFEGRK) of Bacillus subtilis strain ATCC6633 in which the spaB, spaS, spaS-5'-extended and spaI promoter regions that were amplified by PCR are indicated (black and gray boxes on the sizing bar). The terminator sequence detected downstream of the spas gene is indicated as a stem-loop structure. Panel B: Expanded view of the spa-promoter fragments (PspaB, PspaS and PspaI) cloned in pNZ273; indicated are the spaS and spaC coding regions, the spa-boxes and −10 regions within the promoters, as well as the transcription start sites determined in this work and those determined by in previous reports [2,31]. Promoter fragments are aligned on the spa-boxes.

Promoter sequences (pNZ8091 and pNZ8091-2, respectively), or the spaB (pNZ8089) or spaI (pNZ8090) upstream regions (Table 1; Fig. 1). These plasmids were transformed to the wild-type, subtilin-producing B. subtilis strain ATCC6633. All transformants harboring pNZ8089, pNZ8090, pNZ8091 or pNZ8091-2 appeared as blue colonies on plates that contained the chromogenic β-glucuronidase substrate X-Gluc. Moreover, quantitative analysis confirmed that high β-glucuronidase activity levels could be determined in B. subtilis ATCC6633 harboring pNZ8089, pNZ8090, pNZ8091 or pNZ8091-2 (Fig. 2). Furthermore, in all transformants, the β-glucuronidase production appeared to be regulated in a highly similar and typical cell-density-dependent manner, which resembles the production characteristics described for sub-
Growth-phase-dependent (OD 600 curve is given in circles) expression of β-glucuronidase (activity curve is given in triangles and is calculated as a.u. per OD 600) in *Bacillus subtilis* strain ATCC6633 containing the spaB promoter–gusA construct, pNZ8089. The graph shows results obtained from a single experiment, which was chosen as a representative experiment from independent triplicate experiments.

An exemplary growth and β-glucuronidase production curve of the strain harboring pNZ8089 (spaB promoter) is presented in Fig. 3, showing low-level β-glucuronidase activity during the early exponential growth phase, followed by a sharp increase starting approximately at mid-exponential growth phase and eventually reaching maximal activity levels during the beginning of the stationary phase. Remarkably, in these measurements, both plasmids harboring parts of the spaS upstream region (pNZ8091 and pNZ8091-2) did not display any functional difference, while one of these plasmids (pNZ8091-2) lacks the previously determined promoter sequences (see also below).

Subtilin-controlled β-glucuronidase expression

In order to evaluate the subtilin-mediated regulation of the promoters cloned, their activity was analyzed in the chloramphenicol-resistant *B. subtilis* ATCC6633 spaB derivative, which, due to this mutation, has lost the ability to produce subtilin [21]. To this end, the chloramphenicol resistance marker of pNZ273 and its spa-promoter derivatives were replaced by the tetracycline-resistance marker derived from pNZ280 [27]. All transformants of the spaB strain harboring one of the resulting plasmids (pNZ273Tet, pNZ8092, pNZ8093, pNZ8094 or pNZ8094-2; Table 1) remained white on plates containing X-Gluc (data not shown). In analogy, all overnight cultures of these transformants only contained very low levels of β-glucuronidase activity (data not shown). These results show that all DNA fragments cloned represent promoter elements that are only active in the subtilin-producing strain, supporting the suggestion that subtilin acts as an extracellular peptide autoinducer involved in activation of these promoters [16,17,31]. To investigate this possibility, growing cultures of the spaB strain harboring pNZ8092, pNZ8093, pNZ8094, pNZ8094-2 or the vector pNZ273Tet were treated with various amounts of heat-treated supernatants of overnight-grown cultures of the wild-type strain (subtilin containing) or its spaB derivative, and assayed for the induction of β-glucuronidase production. All spa-promoter constructs displayed subtilin-inducible production of β-glucuronidase, confirming the role of this lantibiotic in regulation of these promoter elements. Interestingly, a linear dose–response relationship is observed between the amount of subtilin added and the level of β-glucuronidase activity induced. However, the different spa-promoter constructs displayed a different level of β-glucuronidase production upon treatment with the same amount of subtilin; the β-glucuronidase activity level derived from the spaS promoter constructs (both in pNZ8094 and pNZ8094-2; see also below) appeared to be approximately five-fold higher than that of the spaB promoter construct, which in its turn is approximately two- to three-fold higher than that of the spaI promoter construct (Fig. 4). These results confirm that subtilin acts as an extracellular peptide autoinducer that is involved in activation of its own biosynthesis. Similar to what has been observed in a subtilin-producing strain, cells harboring pNZ8094 displayed the same phenotype as those harboring pNZ8094-2, while the...
former plasmid lacks all previously identified spaS promoter sequences that are present in the latter plasmid. Moreover, virtually identical levels of β-glucuronidase expression were found in cells harboring pNZ8094-2 as compared to those harboring pNZ8094 (Fig. 4), suggesting that the latter plasmid contains all the spaS upstream sequences required for subtilin-mediated promoter control, which appears to contradict previous reports [2,31,32]. To investigate this further, the transcription start site was determined for the corresponding gusA transcripts and was found to be located at a position that corresponds to position −19 relative to the spaS start codon (Fig. 5). This is in clear contrast to the previous mapping of this site at the positions −74 [31] and −223 [2] relative to the start codon. Moreover, the subtilin-mediated regulation of this novel spaS promoter is clearly confirmed, since virtually no primer extension product could be detected in uninduced cells, while the primer extension product intensity appeared to increase following induction with increasing amounts of subtilin (Fig. 5). This novel spaS promoter mapping positions the spa-box at −38 to −23 relative to the transcription start site (Fig. 6), which strongly resembles the gusA position within the spaB promoter (−39 to −24; Fig. 6). In analogy, the transcription start site of the spaI promoter-derived gusA transcript present in the spaB mutant strain harboring pNZ8093 was mapped at position −96 relative to the spa start codon, thereby placing the spa-box within the spaI promoter at −39 to −24 relative to the transcription start site (Fig. 6). Finally, primer extension experiments in cells harboring pNZ8094-2 or pNZ8093 did not confirm the transcription start sites that were previously described [2,31,32].

Fig. 5. Transcription start site (↓) determination of the spaS promoter present in plasmid pNZ8094. Total RNA was isolated from the spaB derivative of Bacillus subtilis ATCC6633 either uninduced or after induction with various amounts of subtilin-containing supernatant (% of total culture volume is indicated). The transcription start site of the spaS promoter derived gusA transcripts (were determined by primer extension using fluorescent primer GUSR (Table 2)). The same primer was used to generate the corresponding sequence reference lanes presented in the top four lanes of the gel, which are continuing to the spaS start codon (bold-italic). The PDR sequences within the spaS promoter sequence that form the spa-box and the −10 box are indicated.

Fig. 6. Sequence alignment of spaB, spaI and spaS promoter sequences described here (panel A) and the structurally related nisA and nisF promoters (panel B) [8]. Previously determined transcription start positions are indicated by an arrow (↓) and transcription start positions determined (spaL and spaS promoters) or confirmed (spaB promoter) in this study are indicated by a symbol (↓↓). The −10 regions (bold-italic) and the PDR sequences (bold-underlined) are indicated. Panel C shows the sequence of the hybrid spars promoter described here, indicated are the −10 (bold-italic) and the spa-box-specific PDR (bold-underlined).
3.3. SpaRK and the spa-box are the only functional units required for subtilin-mediated gene regulation

To analyze whether the subtilin response of the newly identified spaS promoter depends on SpaK and SpaR-mediated signal transduction, plasmid pNZ8094 was transformed to B. subtilis strain 168 that lacks all the spa genes and its derivative NZ8900 in which the spaRK genes have been integrated in the amyE locus. High-level β-glucuronidase production was observed in strain NZ8900 harboring pNZ8094 only when these cells were induced with subtilin (0.131 ± 0.014 a.u. in induced cells versus <0.008 a.u. in uninduced cells). In contrast, neither induced nor uninduced cells of strain 168 harboring pNZ8094 produced significant levels of β-glucuronidase (<0.005 a.u.). These results confirm the role of the subtilin-specific two-component regulatory system in activation of transcription of the spaS promoter, which has been reported to occur through SpaR binding to the spa-box [32].

To establish that the spa-box can directly confer subtilin control to a promoter sequence, the nisin-specific PDR sequences in the nisA promoter (Fig. 6) were replaced by those found in the spa-box. The resulting hybrid promoter was cloned in the gusA promoter probe vector, resulting in pNZ8096, and transformed to the spaB derivative of B. subtilis ATCC6633. The resulting strain did only produce β-glucuronidase when cells were induced with subtilin (0.0105 a.u. in induced cells versus <0.008 a.u. in uninduced cells), while control induction experiments using nisin as an inducer did not render any induction response in these cells. The relative level of β-glucuronidase produced in cells harboring pNZ8096 following subtilin induction appeared to be comparable to that observed for cells harboring pNZ8090, which contains the spaB promoter. Moreover, the control strain harboring pNZ8008 that contains a nisA promoter fusion with gusA did not express significant levels of β-glucuronidase in either the induced or uninduced state (<0.008 a.u.). These results indicate that exchange of the nis-PDR by the spa-PDR generates a concomitant change in lantibiotic response-specificity, thereby clearly confirming the key role of the spa-box in determination of subtilin specific regulatory control. In addition, these results show that the nisin and subtilin regulatory cascades display multi-level specificity that resides in both inducer recognition by the histidine kinase and cis-acting DNA sequence recognition by the response regulator.

4. Discussion

Subtilin is a type I lantibiotic that is produced in a cell-density-dependent manner by B. subtilis. Production of subtilin involves the spa gene cluster encompassing 10 genes, spaFTCSIFEGRK (Fig. 1). Here, we describe the re-mapping of the transcription initiation site of two subtilin-responsive spa-promoters upstream of the spaS and spaI genes. The spaS and spaI promoters identified here are located in closer vicinity to the start codon of the downstream genes as compared to the previously described promoters (Fig. 1) [2,31] and contain the cis-acting element, designated spa-box [32], upstream of their transcription start sites. Remarkably, no functional differences were observed comparing a promoter fragment containing the currently described spaS promoter alone with a 5′-extended version that also includes the previously identified promoter elements upstream of the spaS gene. These findings show that the spaS promoter described here contains all sequence information required for the functional characteristics observed, and indicate that upstream sequences are not required for subtilin-mediated activation of transcription.

The location of the spa-box within the spaS and spaI promoters described here is in analogy with the third subtilin-responsive promoter upstream of the spaB gene [6,31], which is analyzed in parallel by transcriptional fusion to the promoterless gusA gene of E. coli. Thereby, the position of the spa-box within the subtilin-responsive promoters is almost perfectly conserved and appears to replace the canonical –35 region found in Gram-positive promoter sequences. This location of the spa-box upstream of the transcription start site has been described for several bacterial cis-acting enhancer elements that are recognized by transcriptional regulators, including response regulators [1] that share significant homology with SpaR. A clear example is the pho-box in E. coli that is recognized by the response regulator PhoB and is involved in activation of more than 35 genes under phosphate limitation (for reviews, see [33,34]). Recent elucidation of the structure of the PhoB-DNA complex revealed that tandemly arranged PhoB molecules bind to successive direct repeat sequences in the pho-box [4]. The notion that SpaR and PhoB belong to the same family of response regulators and share 35% identity strongly suggests that SpaR binds the spa-box in a similar conformation, and activates spa-promoter activity via a similar mechanism. The relative positioning of the cis-acting spa-box could suggest that the trans-acting regulatory factor, SpaR, directly contacts RNA polymerase to enhance transcription of the downstream genes. However, to date, no experimental evidence is available to support such a direct interaction.

The spaB, spaS and spaI promoters displayed a typical cell-density-dependent activation of transcription activity, initiating around mid-exponential growth and increasing rapidly towards maximum expression levels at the transition to the stationary growth phase. This characteristic corresponds clearly with the transcriptional control of the spa operon and the resulting production kinetics of subtilin [13,16,31,32]. Our results clearly confirm that the regulation of these promoters involves an autoregulatory circuit in which the mature lantibiotic acts as a peptide pheromone that mediates the activation of its own biosynthesis via a two-component regulatory system composed of SpaK and SpaR. Moreover, a linear dose response is observed between the amount of subtilin used for induction and the level of spa-promoter activity. In addition, our results support the notion that the three spa-promoters display a different activity level.
at a specific subtilin concentration. These findings appear to fulfill the requirements for an effective subtilin biosynthesis system, since it is anticipated that multiple subtilin precursors can be handled by a single modification and secretion machinery. Therefore, it is clear that the production machinery encoding spaBThrC transcript is required at lower levels as the structural gene transcript spaC. Nevertheless, since no clear termination sequence is found downstream of the spaC gene, it seems likely that in addition to its own promoter, the spaS transcription level is enhanced by read-through of the spaBThrC promoter. In analogy, the lack of a clear termination sequence downstream of the spaC gene could suggest that the spaD promoter is involved in increased expression of the spaHK promoters under inducing conditions (in presence of the inducer subtilin), which could enhance the spa operon expression even further. Taking these considerations into account indicates that spa gene expression in vivo is potentially more complex than the simplified analysis presented here. Nevertheless, our study clearly establishes three subtilin-dependent promoter elements within the spa gene cluster that undoubtedly play a key role in regulation of subtilin production by B. subtilis. Moreover, the observed differential promoter activity levels observed for the spaS, spaD and spaC promoter constructs is a very good reflection of their relative affinity for the corresponding response regulator SpaR [32], suggesting that the strength of the SpaR-DNA complex directly determines the corresponding response regulator SpaR [32], suggesting that the strength of the SpaR-DNA complex directly determines the differential promoter activity level.

Its regulatory characteristics clearly classify the subtilin biosynthesis system of B. subtilis among the peptide autoinducer-dependent quorum-sensing systems found in various Gram-positive bacteria. A variety of phenotypic traits are regulated by such systems in various Gram-positive hosts, ranging from competence and virulence to type I and type II antimicrobial peptide production (for reviews, see [11,17,18,25]). The highest similarity of the subtilin-dependent promoter elements within the spa gene cluster that undoubtedly play a key role in regulation of subtilin production by B. subtilis. Moreover, the observed differential promoter activity levels observed for the spaS, spaD and spaC promoter constructs is a very good reflection of their relative affinity for the corresponding response regulator SpaR [32], suggesting that the strength of the SpaR-DNA complex directly determines the differential promoter activity level.

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