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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 β -glucuronidase encoding *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,

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enterococci and streptococci. This family of AMPs is characterized by the presence of unusual amino acids like dehydroalanine, dehydrobutyrine and the typical lanthionine or β -methyl-lanthionine bridges.

Biosynthesis of subtilin by *B. subtilis* is dependent on the products of at least 10 genes, i.e. *spaBTCSIFEGRK* 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]. Following 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 SpaI, 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 mid-exponential growth phase and increasing to reach maximal level at the beginning of the stationary phase [13,16,31,32]. Production of subtilin production is regulated at the transcriptional level [2,31,32]. Three promoters have been identified upstream of *spaB*, *spaS* and *spaI*, which drive the regulated transcription of the transcriptional units encompassing *spaBCT*, *spaS* and *spaIFEG*, respectively [16,31,32]. Control of these promoters is mediated by the typical quorum-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 *spaI* 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 *spaI* promoters, and a promoterless copy of the β -glucuronidase-encoding *Escherichia coli gusA* gene. In these constructs, the *spaS* and *spaI* 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 ‘hybrid’ promoter element.

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 μ g/ml, for plasmid encoded and chromosomally encoded resistance marker, respectively), kanamycin (10 μ g/ml) and tetracyclin (10 μ g/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, Mannheim, 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

Table 1
Plasmids used in this study

Plasmid	Relevant characteristics ^a	Reference
pNZ273	Cam ^r , <i>gusA</i> promoter probe vector	[28]
pBTK2	<i>amyE</i> locus integration vector for <i>Bacillus subtilis</i>	^b
pNZ280	Tet ^r , Cam ^r	[27]
pNZ8008	Cam ^r , pNZ273 derivative containing the <i>nisA</i> promoter	[24]
pNZ8024	Cam ^r , pNZ273 derivative containing the <i>nisF</i> promoter	[8]
pNZ273Tet	Tet ^r derivative of pNZ273	This work
pNZ8008Tet	Tet ^r derivative of pNZ8008	This work
pNZ8089	Cam ^r , pNZ273 derivative containing the <i>spaB</i> promoter	This work
pNZ8090	Cam ^r , pNZ273 derivative containing the <i>spaI</i> promoter	This work
pNZ8091	Cam ^r , pNZ273 derivative containing the <i>spaS</i> promoter	This work
pNZ8091-2	Cam ^r , pNZ273 derivative containing the 5' extended <i>spaS</i> promoter [2]	This work
pNZ8092	Tet ^r derivative of pNZ8089	This work
pNZ8093	Tet ^r derivative of pNZ8090	This work
pNZ8094	Tet ^r derivative of pNZ8091	This work
pNZ8094-2	Tet ^r derivative of pNZ8091-2	This work
pNZ8095	Cam ^r , pNZ273 derivative containing the <i>spanis</i> 'hybrid' promoter	This work
pNZ8096	Tet ^r derivative of pNZ8095	This work

^a Cam^r, chloramphenicol resistant; Tet^r, tetracyclin resistant.

^b A generous gift of Dr. S. Bron of the Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands.

Table 2
Primers used in *spa*-promoter amplification, *spanis* hybrid promoter construction, and promoter mapping and sequencing

Primer	Sequence	Position
BF	GCCAAGATCTAGTCCTTTTATGGTATTTACTG	314
BR	AAAAC ^u TCAGAAAAACAATTCTACATCCCTCTGC	456
S1F	GCCAAGATCTTAAAAAAGGAAAAAATGATAAAATCTT	6721
S2F	GCCAAGATCTCCGGACAGGAGTATTTAAGGAAGAGC	6490
SR	AAAAC ^u TCAGCAAATCGAAATCATCGAACTTTGACA	6821
IF	GCCAAGATCTAAATGCTTAAAGTTTCCAGTTGGAA	7379
IR	AAAAC ^u TCAGCCATGAATCCATGTGAAGAATCC	7485
SPANIS	GACCAAGATCTGCTTTGATTAAATTGATAGTTTGTAG	–
PSPARF	CGCAGGATCCGCATGAAATAAATTCAGGGGTATTG	10191
SPAKR	CGAAAGAGCTTCTAGAGAAGATGGATCAG	12420
NISR	GGTCCGGAATTCCTGCAG	–
GUSR*	GGGTTGGGGTTTCTACAGGACGTA	–

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). Primers are designed to introduce *Bgl*III (forward primers), *Pst*I (reverse primers), *Nco*I (SR-TL primer) or *Eco*RI (NISR) restriction sites. Restriction sites are underlined. SPANIS primer is designed for *nisA* promoter mutagenesis, generating the hybrid *spanis* promoter. PSPARF (*Bam*HI) and SPAKR (*Xba*I) primers were used for amplification of the *spaRK* coding sequences and the *spaR* upstream region. GUSR* primer is a fluorescein-labeled primer used for verification of cloned *spa*-promoter sequences and transcription start-site determination.

BR), 100 bp (primer-combination S1F and SR) and 127 bp (primer-combination IF and IR), containing the promoter sequences located upstream of *spaB*, *spaS* and *spaI*, respectively, were amplified. PCR products were purified from the PCR mixture by JETQUICK spin-column purification (ITK-Diagnostics, Amsterdam, The Netherlands), digested with *Bgl*III and *Pst*I, and subsequently cloned in similarly digested pNZ273 [28]. The resulting plasmids, containing *spaB*, *spaI* and *spaS* promoter fragments were designated pNZ8089, pNZ8090 and pNZ8091, respectively. A fourth *spa*-promoter fragment (351 bp, using primers S2F and SR), containing the previously identified *spaS* promoters [2,31,32] as well as the *spaS* promoter identified in this study, was amplified and cloned similar to the three promoter fragments described above; the resulting plasmid was designated pNZ8091-2. Finally, the mutant *nisA* promoter in which the pentanu-

cleotide direct repeat sequences that are typical for the nisin-responsive promoter elements [10,16] were exchanged by those found in the three subtilin-responsive promoter elements, designated *spa*-box (Fig. 6) [32], was constructed by PCR amplification using the mutagenic SPANIS primer in combination with the NISR primer (Table 2) and pNZ8008 [24] as a template. The 228 bp PCR product was digested with *Bgl*III and *Eco*RI and cloned in similarly digested pNZ273. The resulting plasmid that contains the *gusA* gene under control of the 'hybrid-*spanis*' promoter was designated pNZ8095. The chloramphenicol resistance markers of pNZ273 and its derivatives described above and the *nisA* promoter derivative pNZ8008 was removed by *Bgl*III–*Sal*I digestion and replaced by the tetracyclin resistance marker isolated as a *Bam*HI–*Hind*III 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 *tetR* gene was determined by *Bam*HI–*Eco*RI digestion, and those clones that contained the *tetR* gene in opposite orientation relative to the *gusA* gene were used in further experiments. The resulting plasmids were designated pNZ273Tet, pNZ8092 (*spaB* promoter), pNZ8093 (*spaI* promoter), pNZ8094 (*spaS* promoter), pNZ8094-2 (5'-extended *spaS* promoter) and pNZ8096 ('hybrid' *spanis* promoter) (Table 1).

The *spaRK* integration plasmid pNZ8900 was constructed by PCR amplification of the *spaRK* locus, including the *spaR* promoter region, using the primers PPARF and SPAKR (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 pBTK2 (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 autoread 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* NZ8900 (168RK) harboring pNZ8089, pNZ8090, pNZ8091 or pNZ8091-2, grown to an optical density at 600 nm (OD_{600}) of 1.0 and subsequently induced with variable levels of subtilin for 30 min. Primer extension products were analyzed using the ALF DNA sequencer. Raw data from the sequencer were processed using the gel-view module of the corresponding software package.

2.4. Transformation of *B. subtilis* strains

B. subtilis strains were transformed by electroporation. Cells were grown in 2*LB to OD_{600} of 1.0–1.5, and harvested by centrifugation. Cells were subsequently washed with 1 and 0.2 volumes of ice-cold sterile water, and finally resuspended in 0.02 volume of freshly prepared 40 % polyethylene glycol 6000 (PEG-6000, Sigma). Hundred µl of the final cell suspension was mixed with approximately 1 µg of plasmid DNA (volume maximally 3 µl) and electroporated at 1.4 kV, 400 Ohms and 25 µF, using a BIORAD genepulser. 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, trans-

formation efficiencies of 10^3 – 10^4 colony forming units per µg of plasmid DNA were obtained.

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-Gluc; 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 Na_2HPO_4/NaH_2PO_4 , pH 7.0) at a final OD_{600} of 2.0. Cells were permeabilized by adding 50 µl of acetone–toluene (9:1, v/v) per ml of cell suspension and subsequently incubated for 10 min at 37 °C. Forty µl of these permeabilized cells were tested in the β -glucuronidase assay that has previously been described [8]. Expression of β -glucuronidase was induced in the *spaB* derivative of *B. subtilis* ATCC6633 by addition of varying amounts of subtilin containing culture supernatant (heat-treated, 15 min, 85 °C, supernatant of *B. subtilis* ATCC6633) to growing cells at an OD_{600} of 2.0, growth was subsequently continued for 90 min and cells were pelleted by centrifugation and β -glucuronidase activities were analyzed as described above. β -glucuronidase activity is given as the change in absorbance at 405 nm per optical density (OD_{600}) unit of cell suspension (ΔA_{405} , min^{-1} , OD_{600}^{-1} ; arbitrary unit, a.u.). From these arbitrary units, it is possible to calculate the specific β -glucuronidase activity ($nm\ min^{-1}\ OD_{600}^{-1}$) by using the molar absorption coefficient of para-nitrophenyl- β -D-glucuronic acid (18.000).

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 *spaB* 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-

Fig. 1. The *spa*-promoters. Panel A: Schematic representation of the subtilin biosynthesis gene cluster (*spaBTSIFEGRK*) 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 Pspal) 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.

motor 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-

Fig. 2. β -glucuronidase activity levels measured in overnight-grown, permeabilized *Bacillus subtilis* ATCC6633 cells harboring various plasmids (Table 1; Fig. 1). pNZ273 (promoter probe vector) [28], pNZ8089 (*spaB* promoter), pNZ8090 (*spaI* promoter), pNZ8091 (*spaS* promoter), pNZ8091-2 (5'-extended *spaS* promoter).

Fig. 3. Growth-phase-dependent (OD₆₀₀ curve is given in circles) expression of β -glucuronidase (activity curve is given in triangles and is calculated as a.u. per OD₆₀₀) 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.

tilin [2,13,31,32]. 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).

3.2. 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

Fig. 4. β -glucuronidase activity levels measured in permeabilized *Bacillus subtilis* cells of the *spaB* mutant derivative of *B. subtilis* ATCC6633 containing either the *spaS* (pNZ8094, squares), the *spaB* (pNZ8092, circles) or *spaI* (pNZ8093, triangles) promoter derivative of pNZ273Tet. Cells were grown to OD₆₀₀ of ca. 2.0, and subsequently induced for 90 min with different amounts of culture supernatants (indicated as volume fraction of total culture volume) from the subtilin-producing strain *B. subtilis* ATCC6633 (filled symbols) and its *spaB* derivative that does not produce subtilin (open symbols).

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 subtitin-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.

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 subtitin-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 subtitin-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 subtitin (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 *spa*-box 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 *spaI* 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. 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 (*spaI* and *spaS* promoters) or confirmed (*spaB* promoter) in this study are indicated by an arrow and a symbol (↓*). The -10 regions (bold-italic) and the PDR sequences (bold-underlined) are indicated. Panel C shows the sequence of the hybrid *spanis* 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 <0.0008 a.u. in uninduced cells). In contrast, neither induced nor uninduced cells of strain 168 harboring pNZ8094 produced significant levels of β -glucuronidase (<0.0005 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 <0.0008 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 *spaI* 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 (<0.0008 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, *spaBTCSIFEGRK* (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 fulfil 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 *spaBTC* transcript is required at lower levels as the structural gene transcript *spaS*. 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 *spaB* promoter. In analogy, the lack of a clear termination sequence downstream of the *spaG* gene could suggest that the *spaI* promoter is involved in increased expression of the *spaRK* genes 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*, *spaB* and *spaI* 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 relative *spa*-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 autoregulatory system is obviously found in the nisin biosynthesis system of *L. lactis* (for a review, see [16,24,30]). Notably, similar regulatory characteristics were observed for the nisin-responsive promoter elements found within the nisin biosynthetic gene cluster of *L. lactis* [8,9,16]. Moreover, the nisin-responsive promoters identified upstream of the *nisA* and *nisF* genes share a striking degree of structural similarity with the subtilin-responsive *spa*-promoters (Fig. 6), while the response regulators involved in their regulation share a high degree of identity [30]. Exchanging the pentanucleotide direct repeat specifically found in the *nisA* and *nisF* promoters ('*nis-box*': TCTGA-N₆-TCTGA) by the *spa-box* (TTGAT-N₆-TTGAT) rendered a subtilin-responsive promoter element, thereby, further establishing the crucial role of the *spa-box* in subtilin-mediated transcriptional regulation. Overall, the data presented here show that the only essential components required for subtilin-regulated gene expression are the two component regulatory systems (SpaK and SpaR) and the *spa-box* containing promoter. Interestingly, the autoregulatory characteristics of the nisin autoregulatory system have been exploited for the construction of the highly effective and

widely used nisin controlled expression (NICE) system that allows strictly regulated expression of a gene of interest in the original host *L. lactis*, but also in various heterologous Gram-positive hosts [5,9,12,15,26]. The similar autoregulatory characteristics of the subtilin biosynthesis system suggest that this system could be exploited for the construction of a subtilin-regulated expression (SURE) system for *B. subtilis*. Moreover, several of the *spa*-promoter plasmids and *B. subtilis* production hosts (especially NZ8900) described here can be regarded as the first components of such a system.

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