Applied and Environmental Microbiology, Nov. 2006, p. 7270–7277 Vol. 72, No. 11
0099-2240/06/$08.00+0 doi:10.1128/AEM.00723-06

The Lantibiotic Mersacidin Is an Autoinducing Peptide▽

Stephanie Schmitz,[1] Anja Hoffmann,[1] Christiane Szekat,[1] Brian Rudd,[2] and Gabriele Bierbaum[1]*

Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie, Sigmund-Freud-Straße 25, 53105 Bonn, Germany,[1] *and Novacta Biosystems Ltd., UHH Innovation Centre, University of Hertfordshire, Hatfield, Herfordshire AL10 9AB, United Kingdom*[2]

Received 29 March 2006/Accepted 7 September 2006

The lantibiotic (lanthionine-containing antibiotic) mersacidin is an antimicrobial peptide consisting of 20 amino acids and is produced by *Bacillus* sp. strain HIL Y-85,54728. The structural gene (*mrsA*) and the genes for producer self-protection, modification enzymes, transport proteins, and regulator proteins are organized in a 12.3-kb biosynthetic gene cluster on the chromosome of the producer strain. Mersacidin is produced in stationary phase in a synthetic medium (K. Altena, A. Guder, C. Cramer, and G. Bierbaum, Appl. Environ. Microbiol. 66:2565–2571, 2000). To investigate the influence of the alternative sigma factor H on mersacidin biosynthesis, a SigH knockout was constructed. The knockout mutant was asporogenous, and a comparison to the wild-type strain indicated no significant differences concerning mersacidin production and immunity. Characterization of the *mrsA* promoter showed that the gene is transcribed by the housekeeping sigma factor A. The biosynthesis of some lantibiotic peptides like nisin or subtilin is regulated in a cell-density-dependent manner (M. Kleerebezem, Peptides 25:1405–1414, 2004). When mersacidin was added at a concentration of 2 mg/liter to an exponentially growing culture, an earlier production of antibacterial activity against *Micrococcus luteus* ATCC 4698 in comparison to that of the control culture was observed, suggesting that mersacidin itself functions as an autoinducer. In real-time PCR experiments, the expression of *mrsA* was remarkably increased in the induced culture compared to the control. In conclusion, mersacidin is yet another lantibiotic peptide whose biosynthesis can be regulated by an autoinducing mechanism.

Mersacidin is an antimicrobial peptide consisting of 20 amino acids and is produced by *Bacillus* sp. strain HIL Y-85,54728 (5). It belongs to a group of antimicrobial peptides called lantibiotics. These peptides are synthesized by gram-positive bacteria (reviewed in references 23 and 26). Mersacidin exerts its antibacterial activity by inhibition of cell wall biosynthesis; it forms a complex with the peptidoglycan precursor lipid II (4). In contrast to peptide antibiotics like bacitracin or gramicidin S, lantibiotics are ribosomally synthesized and undergo extensive posttranslational modification reactions which lead to the formation of nonproteinogenic amino acids, such as lanthionine and 3-methyllanthionine. Besides lanthionine, lantibiotics contain further rare amino acids, such as didehydroalanine, didehydrobutyrine, and *S*-aminovinylcysteine. Due to their differences in structure, two types of lantibiotics have been distinguished: type A and type B (15). Type A lantibiotics are elongated, flexible peptides that act by forming pores in the bacterial membrane. In contrast, type B lantibiotics are rigid globular peptides with no net charge or a negative net charge. The type B lantibiotics comprise mersacidin, actagardine, and the cinnamycin group of peptides.

The biosynthetic apparatus of a lantibiotic is organized in a biosynthetic gene cluster (26). The biosynthetic gene cluster of mersacidin (12.3 kb) consists of 10 genes and is located on the chromosome of the producer strain *Bacillus* sp. strain HIL Y-85,54728. In addition to the structural gene *mrsA* (3), the gene cluster comprises genes encoding proteins involved in posttranslational modification of the prepeptide, *mrsM* and *mrsD*; a gene coding for a transporter with an associated protease domain, *mrsT*; genes encoding proteins for producer self-protection, *mrsF*, *mrsE*, and *mrsG*; and three genes encoding putative regulatory proteins, *mrsK2*, *mrsR2*, and *mrsR1* (Fig. 1A). Whereas the two-component system MrsR2/K2 is involved in ensuring producer self-protection ("immunity") of the producer strain, the single regulator MrsR1, which lacks a corresponding histidine kinase, is essential for mersacidin biosynthesis (11).

Mersacidin is produced in stationary phase in a synthetic medium (1). Transcription of the structural gene *spaS* of the type A lantibiotic subtilin in *Bacillus subtilis* ATCC 6633 is also associated with stationary phase, and this effect is mediated by the sigma factor H. SigH belongs to a group of alternative sigma factors which are involved in the transcription of specific regulons that are expressed in the later stages of growth (reviewed in references 12 and 20). The protein consists of 218 amino acids and is employed in the transcription of early sporulation genes and genes involved in competence development and antibiotic production (8, 21). The deletion of SigH decreases the expression of subtilin because expression of the regulatory proteins SpaR and SpaK is dependent on SigH (31). After sequencing the mersacidin biosynthetic gene cluster, possible consensus motifs of the housekeeping sigma factors SigA and SigH had been identified in the promoter regions of the structural gene *mrsA* (unpublished data). In order to elucidate the influence of the different sigma factors on mersacidin biosynthesis, a SigH knockout strain was tested for mersacidin production.

In addition to regulation through the activity of sigma factors, other mechanisms have been shown to be involved in

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie, Sigmund-Freud-Straße 25, 53105 Bonn, Germany. Phone: (49) 228-287-19103. Fax: (49) 228-287-14808. E-mail: bierbaum@mibi03.meb.uni-bonn.de.

▽ Published ahead of print on 15 September 2006.

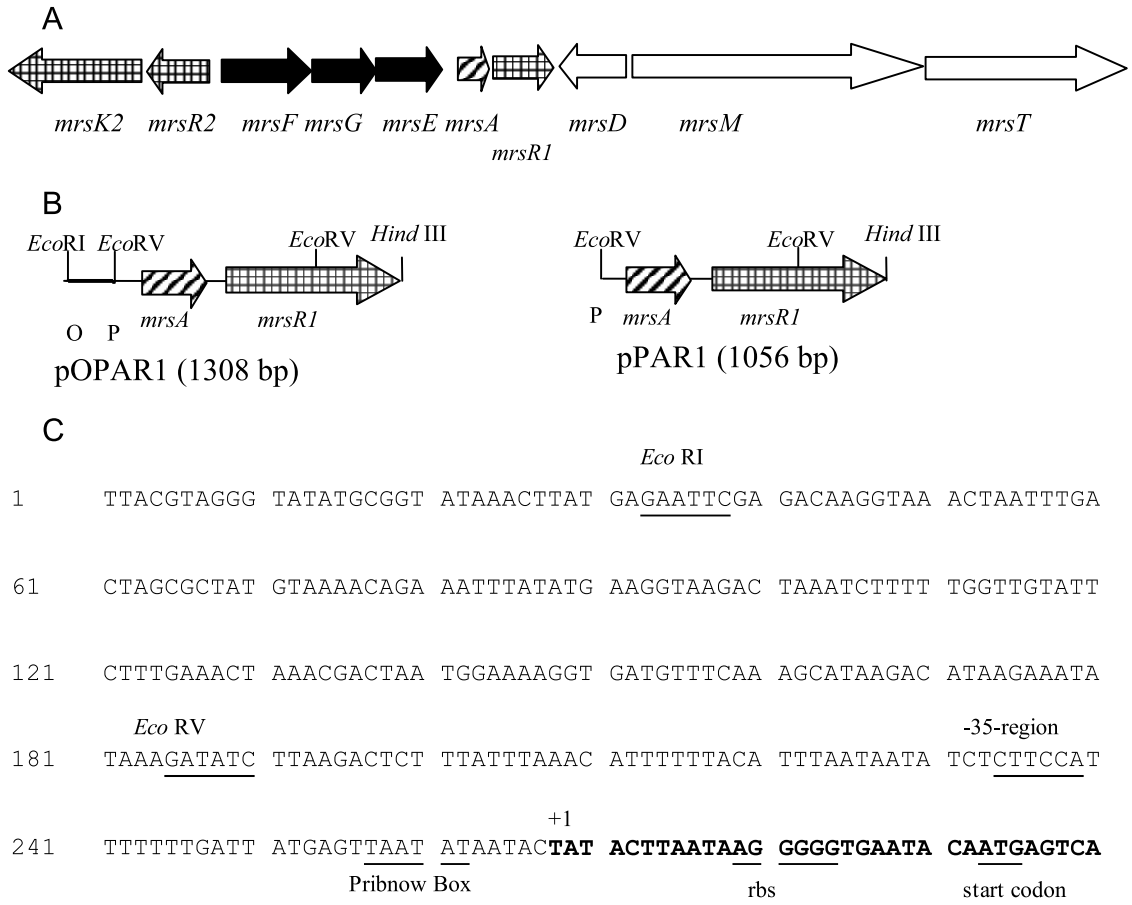

FIG. 1. (A) Mersacidin biosynthesis gene cluster. Organization of the biosynthetic gene cluster of mersacidin, which is located on the chromosome of the producer strain *Bacillus* sp. strain HIL Y-85,54728. Shown are the structural gene (striped arrow), genes necessary for modification and export of mersacidin (white arrows), genes involved in regulation (checkered arrows), and genes for producer self-protection (black arrows). (B) Plasmids pOPAR1 and pPAR1. The plasmids differ in the presence of the putative operator region between the EcoRV and EcoRI sites in plasmid pOPAR1. O stands for operator, P for promoter, A for the structural gene *mrsA* (striped arrows), and R1 for *mrsR1* (checkered arrows). (C) Nucleotide sequence of the promoter region of *mrsA*. Total RNA of the producer strain was isolated, and the 5′ end of the RNA was identified by a 5′ RACE. The start codon, −10 Pribnow box, −35 region, EcoRI and EcoRV restriction sites, and the ribosome binding site (rbs) are underlined. The 5′ end of the mRNA is shown in bold letters.

regulation of lantibiotic biosynthesis. For example, a quorum-sensing mechanism mediates regulation of the biosynthesis of the lantibiotics subtilin and nisin. The peptides act as autoinducers of their own production (reviewed in reference 16). Furthermore, the regulation of various bacteriocins produced by gram-positive bacteria is under control of a cell-density-dependent quorum-sensing mechanism (7, 30, 34). Here we present for the first time the characterization of the *mrsA* promoter and evidence for the regulation of mersacidin biosynthesis by an autoinducing mechanism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains were maintained as glycerol cultures at −70°C and grown on blood agar or in tryptone soy broth. All strains used are listed in Table 1. *Bacillus* sp. strain TTEX was constructed as described previously (33) using an altered sites in vitro mutagenesis system (Serva, Heidelberg, Germany), and a stop codon was introduced in position 4 of the leader peptide employing the primer E4stopmersacidin (Table 2). Appropriate antibiotics (chloramphenicol, 20 mg/liter; and erythromycin, 25 mg/liter) were added to the media used for culturing and selecting mutants. For the detection of mersacidin production, *Bacillus* sp. strain HIL Y-85,54728 and its respective mutants were grown in double-strength synthetic medium (1). Antibacterial activities in the supernatant were determined by agar diffusion assays using the indicator strain *Micrococcus luteus* ATCC 4698. When the producer strains had been cultivated in the presence of chloramphenicol, *Staphylococcus carnosus* TM 300(pTV0MCS), which harbors a chloramphenicol resistance gene, was employed as the indicator strain. *Staphylococcus carnosus* TM 300(pTV0MCS) was incubated overnight at 30°C, *Micrococcus luteus* ATCC 4698 was incubated at 37°C. For determination of bacteriocin production, Mueller-Hinton agar or Columbia blood agar was used.

The plasmid pOPAR1 contains the natural EcoRI-HindIII fragment that harbors the putative operator region upstream of *mrsA*, the promoter, the structural gene *mrsA*, and the gene *mrsR1* (nucleotides 4843 to 6046 of GenBank accession number AJ250862) (Fig. 1B and C). The insert was cut out from the plasmid pMER2, which is a derivative of pMER1 (1), by digestion with EcoRI and HindIII and was ligated into pCU1 (2). The insert of the plasmid pPAR1 starts at the EcoRV site upstream of *mrsA* and was amplified by PCR using primers containing an EcoRI site (primer "5′mrsAR1") and the natural HindIII restriction site (primer "3′mrsAR1") (nucleotides 4998 to 6046 in accession number AJ250862). In contrast to pOPAR1, pPAR1 does not contain the putative operator region between the EcoRI and EcoRV restriction site (Fig. 1B and C). Both plasmids were introduced into *Bacillus* sp. strain HIL Y-85,54728 Rec1.

Isolation of DNA. DNA was prepared using QIAGEN (Hilden, Germany) genomic tips according to the manufacturer's recommendations. General protocols were followed for cloning strategies and enzymatic DNA modifications (27). Digested DNA fragments were eluted from agarose gels employing a MinElute gel extraction kit (QIAGEN).

TABLE 1. Strains used in this study

Strain	Description	Reference or source
Bacillus sp. strain HIL Y-85,54728	Wild-type producer strain of mersacidin	5
Bacillus sp. strain TT	Wild-type producer strain that has been transformed and cured again twice	This study
Bacillus sp. strain TT ΔSIGH1.1 ΔSIGH1.2 ΔSIGH1.3 ΔSIGH1.4	*spo0H* knockout mutants of the mersacidin producer strain	This study
Bacillus subtilis 168 IS20	*spo0H* mutant; *trpC2*	25
Bacillus sp. strain TT Δ*mrsR2/K2*	Δ*mrsR2/K2* knockout mutant	11
Bacillus sp. strain TT Δ*mrsR1*	Δ*mrsR1* knockout mutant	11
Bacillus sp. strain HIL Y-85,54728 Rec1	Inactivation of *mrsA* by an erythromycin resistance cassette by double crossover; no mersacidin production	1
Bacillus sp. strain TTEX	Inactivation of *mrsA* by a stop codon in the leader peptide	This study
Staphylococcus carnosus TM 300	Transformation host	28
Staphylococcus carnosus TM 300(pTV0MCS)	Indicator strain	11
Micrococcus luteus ATCC 4698	Indicator strain	ATCC, Manassas, Va.

Construction of a SigH knockout. Inactivation of the structural gene *spo0H* encoding SigH was performed employing the temperature-sensitive vector pTV0MCS, which carries a chloramphenicol resistance gene (11). *Bacillus* strains and *Staphylococcus carnosus* TM 300 were transformed by protoplast transformation (9, 10). All primers employed are listed in Table 2.

For inactivation of *spo0H*, an internal 339-bp fragment of *spo0H* was amplified by PCR using the primers SigmaH5′ and SigmaH3′. The protein that is encoded by this fragment carries a deletion in the N terminus, which is responsible for binding to the −10 region of the SigH promoter, and a deletion in the C terminus that is involved in binding to the −35 region of the SigH promoter. After digestion with KpnI and XbaI, the fragment was ligated to digested pTV0MCS, which replicates only in gram-positive organisms, and the ligation assay was transformed into *Staphylococcus carnosus* TM 300, yielding pΔSIGH1. The recombinant plasmid was then introduced into *Bacillus* sp. strain TT and integrated into the chromosome by a single crossover. Colonies that had integrated the plasmid into the chromosome were selected by a temperature shift to 42°C in the presence of chloramphenicol (20 mg/liter). The integration of the plasmid was confirmed by PCR using the primer combinations SigH1/pTV0mcsIns-1 and SigH2/pTV0mcsIns-2. The SigH1/2 primers anneal on the chromosome in regions that are not covered by the cloned fragment. The pTV0MCS primers anneal on the plasmid, and the above combinations amplify 596- and 404-bp products, only if the plasmid has been integrated into the chromosome. Loss of the circular form was checked by PCR using pTV0mcsIns-1 and pTV0mcsIns-2, which yields a 496-bp product if circular plasmid is present in the cell. Four knockout strains were chosen for further characterization and were designated *Bacillus* sp. strain TT ΔSIGH1.1, *Bacillus* sp. strain TT ΔSIGH1.2, etc. (Table 1).

TABLE 2. Primers used in this study

Primer	Sequence
5′mrsAR1	5′AGAAATATGAATTCATCTTAAGACTCTTTATTTAAC3′
3′mrsAR1	5′TTGGGTCAAGCTTTTTACACGAC3′
SigmaH5′	5′TATGGTACCATAGGGGCGCACAGAGAGGATA3′
SigmaH3′	5′CTTTCTAGATCTCCCATTTTCATTTCAAT3′
SigH1	5′GTGAATCTACAGAACAAC3′
SigH2	5′GTACTTCTCCAGCTTGCG3′
pTV0mcsIns-1	5′GATTTACATATGAGTTATGCAG3′
pTV0mcsIns-2	5′CGCTCATGGTCAATATCATC3′
RT-5	5′ATTAACAAATACATTCAGAAGTTAGAGTAC3′
oligo-dT-anchor primer	5′GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTV3′[a]
PCR anchor primer	5′GACCACGCGTATCGATGTCGAC3′
RT-12	5′GAGTACAAACACCGCCGCCA3′
RT-4	5′TGAGTCAAGAAGCTATCATTCGTTC3′
5′16S rRNA lang	5′GGGAGCTTGCTCCGATGTTAG3′
3′16S rRNA lang rev	5′CGGCTGGCTCCTAAAAGGTTAC3′
5′16S rRNA	5′TCCGCAATGGACGAAAGTCTGAC3′
3′16S rRNA	5′CCCCAGTTTCCAATGACCCTCC3′
E4stopmersacidin[b]	5′GAATACAATGAGTCAATAAGCTATCATTCGTT3′

[a] V is A, C, or G.
[b] The mutation that leads to the introduction of the stop codon has been underlined.

Determination of sporulation frequency of the knockout mutant. Cultures of the knockout mutants and the wild-type strain were grown in LB medium for at least 48 h at 37°C and 180 rpm. In order to kill vegetative cells and induce spore germination, 1 ml of the cultures was incubated at 90°C for 30 min. The concentrated cell suspension and 10-fold dilutions were plated on nutrient agar and LB (20 mg/liter chloramphenicol). The SigH-deficient strain *B. subtilis* 168 1S20 served as an asporogenous control (25). After 16 h of incubation, the colonies were counted. Microscopic examinations were performed after Gram staining or by phase-contrast microscopy.

Assay of the producer self-protection of the *sigH* knockout mutants. For determination of producer self-protection ("immunity") to mersacidin, the wild-type producer strain *Bacillus* sp. strain HIL Y-85,54728 and the *sigH* knockout mutants were grown to an optical density at 600 nm of 0.3 to 0.5 in 10 ml of half-concentrated Mueller-Hinton broth. Mersacidin was added at a concentration of 10 mg/liter, and growth was monitored at 600 nm.

Mapping of the *mrsA* mRNA using 5′ rapid amplification of cDNA ends (RACE). Isolation of total RNA from *Bacillus* sp. strain HIL Y-85,54728 was performed with a QIAGEN RNeasy midi kit. Before harvesting, the cells were treated with RNAprotect bacteria reagent (QIAGEN, Hilden, Germany) to ensure stability of total RNA. Cultures of the wild-type strain were grown in 15 ml synthetic medium (1) and harvested at an optical density at 600 nm of 2. The cells were lysed by incubation with lysozyme (100 mg/ml) in Tris-EDTA buffer for 20 min at room temperature. The further procedure was performed as described by the manufacturer of the 5′/3′ RACE kit (Roche, Penzberg, Germany). For the 5′ RACE experiment, 0.855 μg of RNA was introduced into the cDNA synthesis using the sequence-specific primer RT-5 in a concentration of 12.5 μM. After a purification step with a HighPure PCR product purification kit (Roche, Penzberg, Germany), the cDNA served as template for a first PCR, using the primers RT-5 and oligo-dT-anchor (Roche, Penzberg, Germany). Since the amount of the resulting PCR product was too small, a second PCR was performed, using the nested primers RT-12 and the PCR anchor primer according to the instructions of the manufacturer (Roche, Penzberg, Germany) (Table 2).

DNA sequence analysis. PCR products were sequenced by Sequiserve (Vaterstetten, Germany). Processing of the nucleotide sequence data was performed by using ClustalW 1.82 at the EMBL website (http://www.ebi.ac.uk/clustalw).

Induction of mersacidin biosynthesis and real-time PCR of *mrsA* transcripts. For the induction experiments, the appropriate strains were cultivated overnight in tryptone soy broth. A portion of this culture (100 μl) served as an inoculum for 10 ml synthetic medium (1), and the cultures were incubated for about 5 h until an optical density at 600 nm of 0.5 to 0.7 had been reached. Mersacidin in a concentration of 2 mg/liter or 2 ml of a sterilized 16-h supernatant was added to the exponentially growing cultures, and the first sample was withdrawn immediately afterwards. Subsequently, growth and production of antibacterial activity

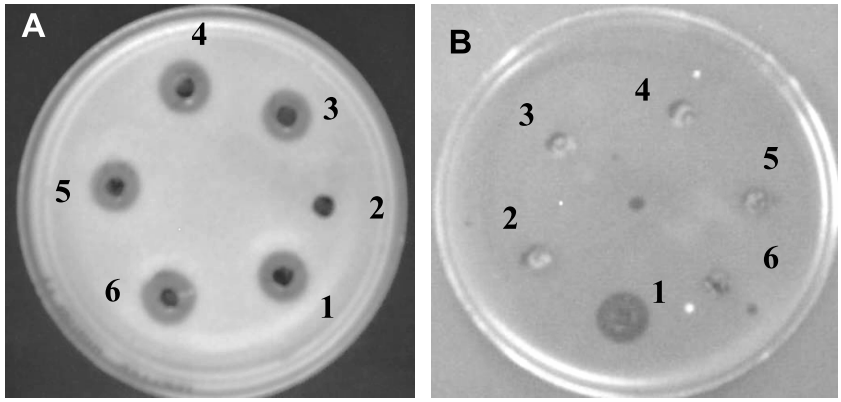

FIG. 2. Agar diffusion assay. (A) Mersacidin production of the wild-type producer strain *Bacillus* sp. strain HIL Y-85,54728 (1), *Bacillus* sp. strain HIL Y-85,54728 Rec1 (2), *Bacillus* sp. strain TT ΔSIGH1.1 (3), *Bacillus* sp. strain TT ΔSIGH1.2 (4), *Bacillus* sp. strain TT ΔSIGH1.3 (5), and *Bacillus* sp. strain TT ΔSIGH1.4 (6) in synthetic medium was analyzed by agar diffusion assay using *Staphylococcus carnosus* TM 300(pTV0MCS) as the indicator strain. (B) Production of bacteriocins other than mersacidin by *Bacillus* sp. strain HIL Y-85,54728 (1), *Bacillus subtilis* 168 1S20 (2), *Bacillus* sp. strain TT ΔSIGH1.1 (3), *Bacillus sp*. strain TT ΔSIGH1.2 (4), *Bacillus* sp. strain TT ΔSIGH1.3 (5), and *Bacillus* sp. strain TT ΔSIGH1.4 (6) in LB using *Micrococcus luteus* ATCC 4698 as the indicator organism.

were monitored by taking aliquots every hour. The culture supernatants employed for induction were obtained after cultivating the wild-type producer or *Bacillus* sp. strain TTEX for 16 h in synthetic medium, which allows mersacidin production (1). The cells were then harvested, and the culture supernatant was sterilized by filtration. The experiments were performed three times.

To confirm the data on a transcriptional level, a real-time PCR experiment was performed by using a LightcyclerFast start DNA Master^{Plus} SYBR green I kit from Roche (Penzberg, Germany). The appropriate strains were cultivated in 25 ml of synthetic medium (1) until an optical density at 600 nm of 0.5 had been reached. After induction with mersacidin at a concentration of 2 mg/liter, the cultures were further incubated for 15 min and 1 h before the total RNA was extracted. The isolated RNA (3 μg) was transcribed into cDNA using the SuperScript II reverse transcriptase from Invitrogen (Karlsruhe, Germany) following the manufacturer's instructions. As a housekeeping gene, the gene encoding the 16S rRNA was amplified by PCR using the primers 5′16S rRNA lang and 3′16S rRNA lang rev. For real-time PCR, the primers 5′16S rRNA and 3′16S rRNA were used for amplification. For the target gene *mrsA*, the primers RT-4 and RT-5 were employed and the plasmid pPAR1 (Fig. 1B) served as a control. PCR products were analyzed on a 2% agarose gel.

Nucleotide sequence accession numbers. The nucleotide sequence of the complete biosynthetic gene cluster of mersacidin is available under GenBank accession number AJ250862. The nucleotide sequence of the gene *spo0H* is available under accession number NC_000964.

RESULTS

Inactivation of *spo0H* and the influence of SigH on mersacidin production and immunity. The transcription factor SigH was inactivated by insertion of the plasmid pΔSIGH1 into its structural gene *spo0H* by a single crossover. For further analysis, four colonies were picked and designated *Bacillus* sp. strain TT ΔSIGH1.1, *Bacillus* sp. strain TT ΔSIGH1.2, etc. In all clones, the correct insertion of pΔSIGH1 into the chromosome of *Bacillus* sp. strain HIL Y-85,54728 was confirmed by two PCR experiments that employ primer combinations which simultaneously anneal on the chromosome and the plasmid and yield only amplification products when the plasmid is inserted in the chromosome. These experiments yielded amplification products of the expected size. In contrast, PCR experiments that rely on circular plasmid as a template did not yield a product, indicating that the plasmid was correctly integrated into the chromosome. After integration of the plasmid, two copies of *spo0H* were present in the chromosome; however, both copies encoded inactive proteins. The upstream copy carries a deletion of the C terminus (bp 489 to 657), and the downstream copy encodes a protein with a deletion in the N terminus (bp 1 to 164). After storage in the cold for 4 days, the colonies showed the typical translucent morphology that had been described by Rogolsky (25) (data not shown). Microscopic examination of the mutant cultured in the presence of chloramphenicol did not show spores, and the absence of spores was also confirmed by heat inactivation of vegetative cells. In contrast to the wild type (1.69×10^8 colonies/ml), the knockout mutants did not form any colonies after 30 min of incubation of the culture at 90°C, indicating that no spores had been formed during growth.

Mersacidin is primarily produced in synthetic medium. Previous experiments with the mersacidin knockout mutant *Bacillus* sp. strain HIL Y-85,54728 Rec1 had shown that the strain is able to excrete at least two other antibacterial substances in this medium; however, these activities were degraded quickly and after 72 h of culture, mersacidin constituted the main antibacterial activity in the supernatant (1, 11). Therefore, the mersacidin production yield of the mutants was analyzed after 72 h of culture in synthetic medium by an agar diffusion assay using *Staphylococcus carnosus* TM 300(pTV0MCS) as an indicator organism. In Fig. 2A, the results of the diffusion assay are summarized. The inhibition zones clearly show that SigH had no influence on final mersacidin yields; in comparison to the wild-type strain, the yield of antibacterial activity by the mutants was not diminished.

In order to analyze a possible influence of SigH on producer self-protection, the wild-type strain and the knockout mutants were cultured in half-concentrated Mueller-Hinton broth. After an optical density of 0.5 had been reached, mersacidin at a concentration of 10 mg/liter was added. After a lag phase of about 3 h, all strains resumed growth. The SigH knockout mutants were not impaired in growth, and no significant differences in susceptibility between the mutants and the wild type could be observed (data not shown).

As mentioned above, the wild-type producer strain is able to

synthesize at least two other substances with antibiotic activity in addition to mersacidin. Antibacterial activity is also produced in complex media, where transcription of *mrsA* is inhibited (A. Hoffmann, unpublished data). To assess production of these other antibacterial substances by the knockout mutants, the strains were grown overnight in a complex medium (Luria-Bertani broth). Examination of the culture supernatants showed that production of these substances was impaired in the mutant strains. Similar results were obtained with a SigH mutant of *B. subtilis* 168 that was included in the test (Fig. 2B). In conclusion, the experiments indicated that the alternative sigma factor H had no influence on production of mersacidin and immunity against mersacidin under the chosen conditions, but it did affect the production of other antibacterial substances by the producer strain.

Characterization of the *mrsA* promoter and the influence of a putative operator region on *mrsA* transcription. In order to map the *mrsA* mRNA, a 5′ RACE experiment was performed. The 5′ end of the *mrsA* mRNA is indicated in Fig. 1C. A possible Pribnow box (−10 region) of a SigA-dependent promoter with four matches to the consensus sequence (TATAAT) is located 31 bp upstream of the start codon ATG and 5 bp upstream of the +1 position of the *mrsA* mRNA. A tentative −35 region with three matches to the consensus −35 sequence (TTGACA) is located 53 bp upstream of the ATG codon (Fig. 1C). In between there is a characteristic spacer of 17 bp. The consensus sequences indicate that *mrsA* is most probably transcribed by the housekeeping SigA-dependent RNA polymerase. The upstream region of the promoter contains an AT-rich sequence between positions −36 and −70 that is typical for the upstream regions of *Bacillus subtilis* SigA promoters and may contact the α-unit of the RNA polymerase (14).

In order to analyze whether an operator region is localized upstream of the *mrsA* promoter, *Bacillus* sp. strain HIL Y-85,54728 Rec1 (1), which harbors an erythromycin resistance cassette instead of the structural gene *mrsA*, was transformed with the plasmids pPAR1 (carrying *mrsA*, *mrsR1*, and the promoter of *mrsA*) and pOPAR1 (harboring an insert that starts 235 bp upstream of the transcriptional start site and includes the promoter and the putative operator sequence in addition to *mrsA* and *mrsR1*) (Fig. 1B). The transcription of *mrsA* was analyzed in the clones and the wild-type strain in real-time PCR experiments. The 16S rRNA served as a standard housekeeping gene. The transcription rates of the wild-type producer, *Bacillus* sp. strain HIL Y-85,54728 ($5.1 \times 10^5 \pm 3.1 \times 10^5$ copies/10^6 copies 16S rRNA), and *Bacillus* sp. strain HIL Y-85,54728 Rec1(pOPAR1) ($4.33 \times 10^5 \pm 2.9 \times 10^5$ copies/10^6 copies of 16S rRNA) were in a similar range, whereas the transcription of *mrsA* in *Bacillus* sp. strain HIL Y-85,54728 Rec1(pPAR1) ($5.16 \times 10^3 \pm 1.6 \times 10^3$ copies/10^6 copies of 16S rRNA) was 100-fold decreased. These data show that this region has a positive influence on transcription of *mrsA* and indicate that a putative operator region, which may serve as a binding site of at least one regulatory protein, or a second promoter exists upstream of the SigA-dependent promoter.

Mersacidin biosynthesis can be induced. To analyze whether mersacidin biosynthesis can be induced, induction experiments employing pure mersacidin as well as 16-h culture supernatant were performed with *Bacillus* sp. strain HIL Y-85,54728 as described in Materials and Methods. The 16-h supernatant of the producer strain used in these experiments contained 10.6 ± 1.9 mg/ml of mersacidin as well as any other molecule that might be involved in induction of mersacidin biosynthesis, since after 16 h of culture, mersacidin production is well under way (1). After the addition of 2 mg/ml mersacidin, as well as after the addition of 2 ml of sterilized supernatant to 10 ml of culture (introducing about 1.8 mg/ml mersacidin), a production of antibacterial activity that was earlier than that of the control culture could be detected (Fig. 3A).

However, the producer strain is able to excrete several substances with antibacterial action. To show that the antibacterial activity in the induced culture supernatants is elevated because of the production of mersacidin, the same experiment was performed with one of the SigH mutants, which—as shown above—is impaired in production of antibacterial substances other than mersacidin. After the induction with mersacidin and 16-h supernatant of *Bacillus* sp. strain HIL Y-85,54728, an earlier production of antibacterial activity could also be observed with the knockout mutant (Fig. 3 B). Since the effect was mediated not only by culture supernatant but also by pure mersacidin in a comparable concentration, the result indicated that mersacidin may be the active component in the 16-h supernatant and may be recognized by the cell.

The next question was whether this mechanism is based on specific recognition of the mersacidin molecule itself, i.e., possibly mediated by the specific two-component regulatory system MrsR2/MrsK2, which is encoded in the mersacidin biosynthetic gene cluster, or more unspecifically due to the action of mersacidin on the cell, i.e., inhibition of cell wall biosynthesis by complexing lipid II (4). Therefore, another inhibitor of cell wall biosynthesis, the glycopeptide antibiotic vancomycin, was added in a subinhibitory concentration (0.5× MIC) to an exponentially growing culture. The mode of action of vancomycin is very similar to that of mersacidin: both compounds complex lipid II, thereby inhibiting transglycosylation. After the addition of vancomycin, no earlier onset of production could be detected in comparison to that of the control (Fig. 3C), indicating that inhibition of cell wall biosynthesis by complexing lipid II is not sufficient to induce mersacidin biosynthesis.

To make sure that it was not a metabolic by-product or other antibacterial substance excreted by the producer strain that was involved in induction, 16-h supernatant of *Bacillus* sp. strain TTEX, a producer strain which carries a point mutation in the mersacidin structural gene introducing a stop codon after the first three amino acids of the leader peptide, was prepared and tested for induction. The TTEX supernatant was inactive in the experiment, which further underlines that the induction is directly dependent on mersacidin (Fig. 3C).

In order to define the minimum concentration of mersacidin needed for induction, lower mersacidin concentrations were tested. However, an induction of mersacidin production after the addition of 0.25 mg/liter and 0.5 mg/liter of mersacidin was not detectable (Fig. 3C). Experiments using 1 mg/ml sometimes showed a slight effect (data not shown).

To confirm the results observed above on the transcriptional level, quantitative real-time PCR experiments were performed. Figure 4 shows the transcription rates after the induction of

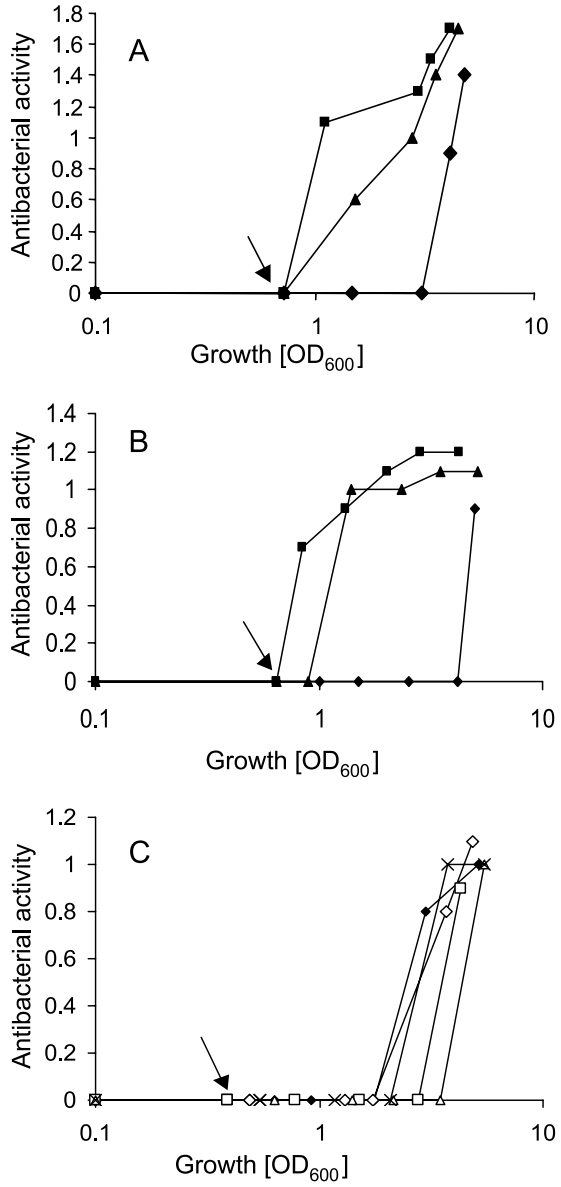

FIG. 3. Induction of the production of antibacterial activity by mersacidin. Growth and production of antibacterial activity by *Bacillus* sp. strain HIL Y-85,54728 (A, C) or *Bacillus* sp. strain TT ΔSIGH1.1 (B). In panels A and B, the antibacterial activity produced by the cells after the addition of 2 ml of sterilized 16-h supernatant (■), or pure mersacidin at a concentration of 2 mg/liter (▲), and the control (no addition) (◆) are plotted against the growth of the cultures. In panel C, production of antibacterial activity by *Bacillus* sp. strain HIL Y-85,54728 after addition of vancomycin (0.5× MIC) (□), mersacidin (0.25 mg/liter) (×), mersacidin (0.5 mg/liter) (◇), 16-h supernatant of *Bacillus* sp. strain TTEX (Δ), and the control (no addition) (◆) are plotted against the optical density (OD_{600}) of the cultures. *Micrococcus luteus* ATCC 4698 was used as the indicator strain in an agar diffusion assay. The addition of mersacidin/vancomycin or supernatant is marked by an arrow.

exponentially growing cultures with mersacidin in a concentration of 2 mg/liter. After addition of mersacidin, the wild-type strain indeed showed an increased expression of the structural gene at 1 h postinduction. This result strongly supports the previous observations and suggests that an autoinducing mechanism is involved in mersacidin biosynthesis. Knockout mutants in the regulatory proteins of the mersacidin gene cluster were also included in this test. In *Bacillus* sp. strain TT Δ*mrsK2/R2*, the two-component regulatory system, MrsR2 and MrsK2, has been inactivated by insertion of an erythromycin cassette (11). In contrast to the wild-type producer, this strain did not increase transcription of the mersacidin mRNA, indicating that the two-component regulatory system is involved in the recognition of the signal and regulation of transcription of *mrsA*. In *Bacillus* sp. strain TT Δ*mrsR1*, the single regulatory protein MrsR1 has been inactivated (11). This strain showed a very slightly increased expression of *mrsA* after induction (Fig. 4).

DISCUSSION

After depletion of one or more nutrients in the environment, *Bacillus subtilis* cells enter stationary phase. This leads to the expression of a wide variety of genes that encode functions in alternative pathways of metabolism, energy production, and development (32). These pathways involve highly interconnected regulatory systems, which control sporulation, genetic competence, synthesis of degradative enzymes, chemotaxis, and the synthesis of antibiotics (13, 22). One factor, which is involved in sporulation and antibiotic production, is the alternative sigma factor H, which is encoded by the gene *spo0H* (12). The production of the peptide antibiotic gramicidin S is under control of a SigH-dependent RNA polymerase (21). For the lantibiotic subtilin, which is produced by *Bacillus subtilis* ATCC 6633, SigH has an influence on production of the peptide. A SigH deletion mutant showed a strongly decreased subtilin production, while the production in an AbrB-deficient background was increased (31). The results presented here demonstrate that inactivation of the alternative sigma factor H had no influence on the yield of mersacidin in synthetic medium. In addition, the producer self-protection ("immunity") against mersacidin was not diminished. However, production of antibacterial substances other than mersacidin was markedly impaired (Fig. 4B). Characterization of the promoter of the structural gene *mrsA* confirmed the above result and suggested that the gene is under control of a SigA-dependent RNA polymerase. Furthermore, the presence of sequences located upstream of the promoter led to an activation of transcription in the producer strain, indicating that an operator might be located in this area.

The biosynthesis of bacteriocins has to be carefully regulated, since it is associated with considerable energy expense for the producer strains. For example, during biosynthesis of the lantibiotic lactocin S, which is produced by *Lactobacillus sakei* L45, the protein LasX acts both as activator and as repressor. It activates the transcription of the long transcript covering *lasA* to *lasW* and negatively regulates the transcription of the *lasXY* operon (24). Two proteins, CylR1 and CylR2, are involved in the regulation of biosynthesis of the two-component lantibiotic cytolysin. The cytolysin operon is repressed by the activity of these proteins and derepressed by an ingenious quorum-sensing mechanism that is activated only in the presence of target cells (30). Especially, cell-density-dependent quorum-sensing mechanisms are widespread in lantibiotic biosynthesis, e.g., subtilin (17), nisin (19), streptin (35), and sali-

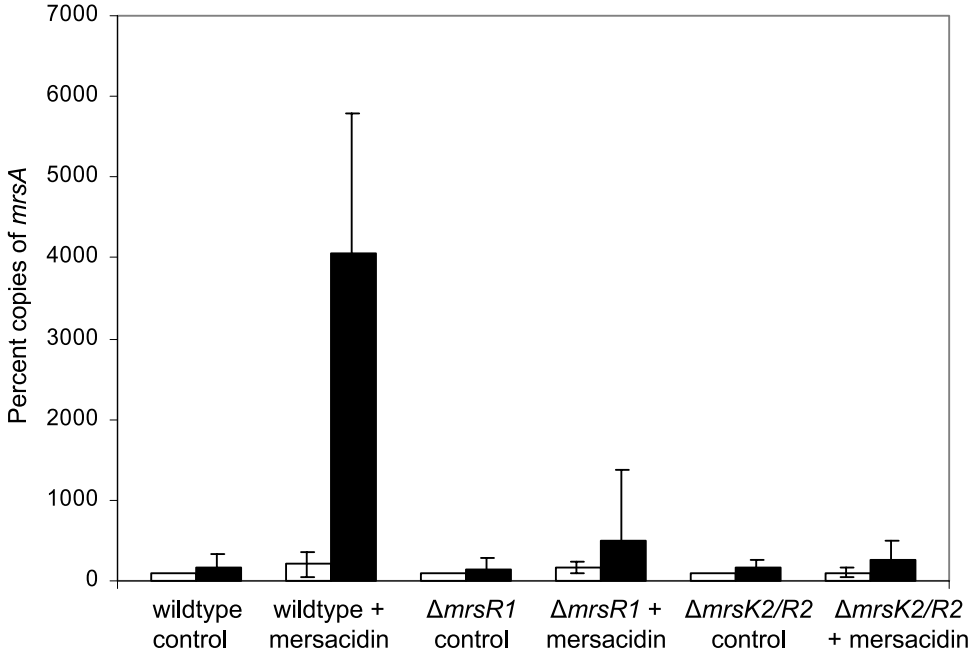

FIG. 4. Influence of an autoinducing mechanism in mersacidin biosynthesis. Percentage of copies of *mrsA* per 10^6 copies of 16S rRNA after addition of mersacidin (2 mg/liter) to the wild-type producer (*Bacillus* sp. strain HIL Y-85,54728) and knockout mutants (*Bacillus* sp. strain TT Δ*mrsR2*/*K2* and *Bacillus* sp. strain TT Δ*mrsR1*). The strains were grown to an optical density at 600 nm of 0.5, mersacidin was added, and aliquots were withdrawn after 15 min (white bars) and after 60 min (black bars) for real-time PCR. Controls show the transcript levels of cultures grown in the absence of mersacidin. The transcript levels of the controls (no addition) measured 15 min after addition of mersacidin to the test cultures were set to 100% transcription.

varicin A (34) autoregulate their own biosyntheses. Here we show that mersacidin is able to induce its own biosynthesis. When mersacidin was added to an exponentially growing culture of the producer strain, i.e., before the start of mersacidin biosynthesis, transcription of *mrsA* markedly increased after the addition of the peptide. This activation mechanism seemed specific for addition of mersacidin. Vancomycin is a glycopeptide antibiotic that—similar to mersacidin—exerts its antimicrobial activity by binding to lipid II. However, vancomycin did not activate *mrsA* transcription, indicating that the activation is not caused by inhibition of cell wall biosynthesis by complexing lipid II.

The mersacidin biosynthesis gene cluster contains two regulatory proteins, MrsR1 and MrsR2, and a histidine kinase, MrsK2. For MrsR1 and the MrsR2/K2 system, knockout mutants have been constructed. Knockout of the single regulator MrsR1 inhibited production of mersacidin in synthetic medium. In contrast, knockout of the two-component system MrsR2/K2 did not affect the yield of the mersacidin fermentation but exerted a pronounced effect on the producer self-protection against mersacidin (11). Here, transcription of *mrsA* in early exponential phase was tested in both knockout strains in the presence of mersacidin. Inactivation of *mrsR2*/*K2* clearly inhibited the induction of transcription by mersacidin. Therefore, the MrsR2/K2 two-component system is probably involved in the recognition of mersacidin in the supernatant, and the sensor kinase MrsK2 may function as signal transducer. In the MrsR1 knockout mutant, the activation of transcription was also strongly decreased, indicating that MrsR1 may be involved in regulation of transcription of *mrsA* as well. With regard to earlier experiments, the effect of the inactivation of MrsR2/K2 was a surprising result, since the Δ*mrsR2*/*K2* mutant nevertheless is able to synthesize mersacidin (11). After sequencing the promoter and the putative operator region of the knockout mutant, no mutations were observed compared to the wild type. In consequence, induction of transcription of *mrsA* by mersacidin cannot be an essential mechanism and most probably only serves to synchronize the biosynthetic effort of the culture. The main function of the system probably resides in the induction of the producer self-protection, i.e., transcription of the ABC transporter MrsFGE (11). Another contrast to the systems mentioned above is that very low concentrations of nisin are effective in inducing production (19), whereas relatively high concentrations of mersacidin had to be employed here. This fact may indicate that production in vivo occurs under conditions of high population densities where a high threshold concentration will be reached quickly, e.g., in a surface-attached biofilm. The availability of the asporogenous SigH mutant has made it possible to perform deferred antagonism tests with colonies on production agar. After culture on production agar, very large inhibition zones were observed, indicating that the production of mersacidin might be enhanced on solid media. On the other hand, the relatively high concentrations that were needed for induction may again indicate that the main function of this mechanism is the induction of producer self-protection or "immunity." This hypothesis is emphasized by the fact that the signal transduction system MrsR2/K2 is essential for the full development of immunity (11). When the expression of the immunity proteins MrsFGE is inhibited, the MIC of the producer strain lies at about 5

mg/ml mersacidin. Therefore, a threshold concentration of 1 to 2 mg/ml mersacidin, which will activate transcription of the immunity genes just in time to avoid a growth inhibition, seems plausible. Similar mechanisms have been described for the immunity proteins of other lantibiotics: the promoter of the gene *nisF* is nisin inducible (6) and the promoter of *spaI*, which confers immunity against the type A lantibiotic subtilin, exhibited quorum-sensing activity in the producer strain *Bacillus subtilis* ATCC 6633 (18).

Mersacidin is an antibiotic with good activity against methicillin-resistant staphylococci, and a system for site-directed mutagenesis of mersacidin has been established in the wild-type producer strain (33). Successful production of mersacidin mutant peptides with high antibacterial activity will require an efficient expression of producer self-protection. The perception of mersacidin as an autoinducing peptide is therefore an important step towards the development of novel antibiotics.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (grant Bi 504/1-3) and Novacta Biosystems Limited.

We are grateful to Sanofi-Aventis, Frankfurt, Germany, for providing the mersacidin producer strain.

REFERENCES

1. **Altena, K., A. Guder, C. Cramer, and G. Bierbaum.** 2000. Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. Appl. Environ. Microbiol. **66:**2565–2571.
2. **Augustin, J., R. Rosenstein, B. Wieland, U. Schneider, N. Schnell, G. Engelke, K.-D. Entian, and F. Götz.** 1992. Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. Eur. J. Biochem. **204:**1149–1154.
3. **Bierbaum, G., H. Brötz, K. P. Koller, and H. G. Sahl.** 1995. Cloning, sequencing and production of the lantibiotic mersacidin. FEMS Microbiol. Lett. **127:**121–126.
4. **Brötz, H., G. Bierbaum, P. E. Reynolds, and H. G. Sahl.** 1997. The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. Eur. J. Biochem. **246:**193–199.
5. **Chatterjee, S., S. Chatterjee, S. J. Lad, M. S. Phansalkar, R. H. Rupp, B. N. Ganguli, H. W. Fehlhaber, and H. Kogler.** 1992. Mersacidin, a new antibiotic from *Bacillus*: fermentation, isolation, purification and chemical characterization. J. Antibiot. **45:**832–838.
6. **De Ruyter, P. G. G. A., O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. De Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. **178:**3434–3439.
7. **Eijsink, V. G., L. Axelsson, D. B. Diep, L. S. Havarstein, H. Holo, and I. F. Nes.** 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Leeuwenhoek **81:**639–654.
8. **Errington, J.** 1996. Determination of cell fate in *Bacillus subtilis*. Trends Genet. **12:**31–34.
9. **Götz, F., and B. Schumacher.** 1987. Improvement of protoplast transformation in *Staphylococcus carnosus*. FEMS Microbiol. Lett. **40:**285–288.
10. **Grosch, J. C., and K. L. Wollweber.** 1982. Transformation of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* protoplasts by plasmid DNA, p. 97–105. *In* U. N. Streips, H. S. Goodgal, W. R. Guild, and G. A. Wilson (ed.), Genetic exchange. Marcel Dekker, New York, N.Y.
11. **Guder, A., T. Schmitter, I. Wiedemann, H. G. Sahl, and G. Bierbaum.** 2002. Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. Appl. Environ. Microbiol. **68:**106–113.
12. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59:**1–30.
13. **Hamoen, L. W., H. Eshuis, J. Jongbloed, G. Venema, and D. van Sinderen.** 1995. A small gene, designated *comS*, located within the coding region of the fourth amino acid-activation domain of *srfA*, is required for competence development in *Bacillus subtilis*. Mol. Microbiol. **15:**55–63.
14. **Helmann, J. D.** 1995. Compilation and analysis of Bacillus subtilis σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
15. **Jung, G.** 1991. Lantibiotics—ribosomally synthesized biologically active polypeptides containing sulphide bridges and α, β-didehydroamino acids. Angew. Chem. Int. Ed. Engl. **30:**1051–1068.
16. **Kleerebezem, M., W. M. de Vos, and O. P. Kuipers.** 1999. The lantibiotics nisin and subtilin act as extracellular regulators of their own biosynthesis, p. 159–174. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signalling in bacteria. ASM Press, Washington, D.C.
17. **Kleerebezem, M.** 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides **25:**1405–1414.
18. **Kleerebezem, M., R. Bongers, G. Rutten, W. M. de Vos, and O. P. Kuipers.** 2004. Autoregulation of subtilin biosynthesis in *Bacillus subtilis*: the role of the *spa*-box in subtilin-responsive promoters. Peptides **25:**1415–1424.
19. **Kuipers, O. P., M. M. Beerthuyzen, P. G. G. A. de Ruyter, E. J. Luesink, and W. M. de Vos.** 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. **270:**27299–27304.
20. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. **174:**3843–3849.
21. **Marahiel, M. A., M. M. Nakano, and P. Zuber.** 1993. Regulation of peptide antibiotic production in *Bacillus*. Mol. Microbiol. **7:**631–636.
22. **Msadek, T.** 1999. When the going gets tough: survival strategies and environmental signalling networks in *Bacillus subtilis*. Trends Microbiol. **7:**201–207.
23. **Pag, U., and H.-G. Sahl.** 2002. Multiple activities in lantibiotics—models for the design of novel antibiotics? Curr. Pharm. Design **8:**815–833.
24. **Rawlinson, E. L., I. F. Nes, and M. Skaugen.** 2002. LasX, a transcriptional regulator of the lactocin S biosynthetic genes in *Lactobacillus sakei* L45, acts both as an activator and a repressor. Biochimie **84:**559–567.
25. **Rogolsky, M.** 1969. Chromosomal regions which control sporulation in *Bacillus subtilis*. Can. J. Microbiol. **15:**787–790.
26. **Sahl, H. G., and G. Bierbaum.** 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. Annu. Rev. Microbiol. **52:**41–79.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Schleifer, K. H., and U. Fischer.** 1982. Description of a new species of the genus *Staphylococcus*: *Staphylococcus carnosus*. Int. J. Syst. Bacteriol. **32:**153–156.
29. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Götz, and K. D. Entian.** 1992. Analysis of genes involved in biosynthesis of lantibiotic epidermin. Eur. J. Biochem. **204:**57–68.
30. **Shankar, N., P. Coburn, C. Pillar, W. Haas, and M. Gilmore.** 2004. Enterococcal cytolysin: activities and association with other virulence traits in a pathogenicity island. Int. J. Med. Microbiol. **293:**609–618.
31. **Stein, T., S. Borchert, P. Kiesau, S. Heinzmann, S. Kloos, C. Klein, M. Helfrich, and K. D. Entian.** 2002. Dual control of subtilin biosynthesis and immunity in *Bacillus subtilis*. Mol. Microbiol. **44:**403–416.
32. **Strauch, M. A., and J. A. Hoch.** 1993. Signal transduction in *Bacillus subtilis* sporulation. Curr. Opin. Genet. Dev. **3:**203–212.
33. **Szekat, C., R. W. Jack, D. Skutlarek, H. Färber, and G. Bierbaum.** 2003. Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. Appl. Environ. Microbiol. **69:**3777–3783.
34. **Upton, M., J. R. Tagg, P. Wescombe, and H. F. Jenkinson.** 2001. Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. J. Bacteriol. **183:**3931–3938.
35. **Wescombe, P. A., and J. R. Tagg.** 2003. Purification and characterization of streptin, a type A1 lantibiotic produced by *Streptococcus pyogenes*. Appl. Environ. Microbiol. **69:**2737–2747.