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# Biosynthesis of the Lantibiotic Subtilin Is Regulated by a Histidine Kinase/Response Regulator System

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**Subtilin is a lanthionine-containing peptide antibiotic (lantibiotic) which is produced by *Bacillus subtilis* ATCC 6633. Upstream from the structural gene of subtilin, *spaS*, three genes (*spaB*, *spaT*, and *spaC*) which are involved in the biosynthesis of subtilin have been identified (C. Klein, C. Kaletta, N. Schnell, and K.-D. Entian, Appl. Environ. Microbiol. 58:132–142, 1992). By using a hybridization probe specific for these genes, the DNA region downstream from *spaS* was isolated. Further subcloning revealed a 5.2-kb *Kpn*I-*Hin*dIII fragment on which two open reading frames, *spaR* and *spaK*, were identified approximately 3 kb downstream from *spaS*. The *spaR* gene encodes an open reading frame of 220 amino acids with a predicted molecular mass of 25.6 kDa. SpaR shows 35% similarity to positive regulatory factors OmpR and PhoB. The *spaK* gene encodes an open reading frame of 387 amino acids with a predicted molecular mass of 44.6 kDa and was highly similar to histidine kinases previously described (PhoM, PhoR, and NtrB). Hydrophobicity blots suggested two membrane-spanning regions. Thus, *spaR* and *spaK* belong to a recently identified family of environmentally responsive regulators. These results indicated a regulatory function of *spaR* and *spaK* in subtilin biosynthesis. Indeed, batch culture experiments confirmed the regulation of subtilin biosynthesis starting in the mid-logarithmic growth phase and reaching its maximum in the early stationary growth phase. Gene deletions within *spaR* and *spaK* yielded subtilin-negative mutants, which confirms that subtilin biosynthesis is under the control of a two-component regulatory system. A fusion of the *spaR* open reading frame with the constitutively expressed *spo* promoter was used to complement the *spaR* mutant to exclude any polar effects of *spaR* on *spaK*.**

Subtilin is a lanthionine-containing peptide antibiotic (lantibiotic) produced by *Bacillus subtilis* ATCC 6633. Lantibiotics have high antimicrobial activity against several gram-positive bacteria such as *Propionibacterium acnes*, staphylococci, streptococci, and clostridia. They contain the unusual amino acids dehydroalanine and dehydrobutyrine and have been termed lantibiotics because of their characteristic sulfide bridges consisting of *meso*-lanthionine and 3-methyl-lanthionine (47). Because of their chemical structure, lantibiotics can be divided into two subgroups (21): (i) linearly shaped lantibiotics including subtilin (17), nisin (33, 34, 39), epidermin (1, 2), gallidermin (26), and Pep5 (40) and (ii) globularly shaped lantibiotics such as cinnamycin (same as Ro 09-0198, which is the same as lanthiopeptin) (7, 27, 28, 36), duramycin (16), and ancovenin (53).

Subtilin is very similar to nisin, which is the most important member of the group of linear lantibiotics and is used as a food preservative. Nisin occurs naturally in dairy products (11) and can be used to prevent growth of clostridia in cheese and canned food. Since the realization of the mutagenic effect of nitrite, which is mainly used against clostridia in canned food, there is an increasing interest in the use of nisin as a replacement (39). We have chosen subtilin as a model system for lantibiotic biosynthesis because its producer, *B. subtilis*, is very suitable for genetic and biochemical investigations.

As first described for epidermin, lantibiotics are ribosomally synthesized as prepeptides and posttranslationally modified (46). The primary transcript of lantibiotic genes is a prepeptide which consists of an N-terminal leader sequence followed by a C-terminal propeptide from which the lantibiotic is matured. A similar gene structure was also found for the linear lantibiotics subtilin (6), nisin (10, 14, 22), gallidermin (46), and Pep5 (24) and the globular lantibiotic cinnamycin (23). The mature peptide contains the unusual amino acids lanthionine, dehydroalanine, and dehydrobutyrine. The formation of these unusual amino acids could be explained by posttranslational dehydration of peptide serine and threonine residues, with subsequent addition of cysteine sulfur to these dehydroamino acids (19, 20).

Assuming that these biosynthetic genes are localized adjacent to the structural gene, we cloned the region upstream from the subtilin structural gene (*spaS*). Sequencing analysis revealed the open reading frames *spaB*, *spaT*, and *spaC*. Gene disruption experiments showed that these genes are essential for subtilin biosynthesis (25, 29). Whereas *spaB* and *spaC* might encode enzymes necessary for posttranslational modification of the subtilin prepeptide, *spaT* is obviously responsible for transport. These genes showed significant homologies to open reading frames identified near the structural genes of the lantibiotics epidermin (45, 47) and nisin (15).

In the present paper, we report on the regulation of subtilin biosynthesis. We could identify two genes, *spaR* and *spaK*, which are involved in the regulation of subtilin biosynthesis. Both genes are located about 3 kb downstream from the subtilin structural gene (*spaS*). SpaR and SpaK belong to a new family of regulatory proteins which has been identified in the past few years. We show that SpaR and SpaK share sequence homology with other two-component regulator pairs. Deletions of either the *spaR* or the *spaK* gene resulted in subtilin-negative mutants. Our results proved that SpaR and SpaK are essential for subtilin biosynthesis and provide the genetic basis for improving the biosynthesis of subtilin.

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## MATERIALS AND METHODS

**Strains and media.** *B. subtilis* ATCC 6633 was used as the subtilin-producing strain. Recombinant plasmids were amplified in *Escherichia coli* RR1 ($F^-$ *hsd520 supE44 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*). *Micrococcus luteus* ATCC 9341 was used as a test strain in halo assays. *E. coli* and *M. luteus* were grown on Luria-Bertani medium (GIBCO, Neu-Isenburg, Germany). *B. subtilis* was grown on TY medium (0.8% tryptone, 0.5% yeast extract [Difco, Detroit, Mich.], 0.5% NaCl). In the case of the use of resistance markers, 10 μg of kanamycin and 5 μg of chloramphenicol per ml were added to the medium. For *B. subtilis* transformation, HS medium was used as described previously (29).

**Plasmids.** A 7.7-kb *Hin*dIII fragment containing *spaR* and *spaK* was subcloned in plasmid pUC19 (54) to yield pCK31. For DNA sequencing and gene deletion experiments, subclones were made in *E. coli* vector pUC19. For complementation of the *spaR* mutant, a shuttle vector containing the *Eco*RI fragment of pEB112 (30) in pBluescript $II^R$ (Stratagene, Heidelberg, Germany) was constructed and the *spaR* gene was fused to the constitutively expressed *spo* promoter (pCE40).

**Molecular biology techniques.** Established protocols were followed for molecular biology techniques (41). DNA was cleaved according to the conditions recommended by the commercial supplier of the restriction enzymes (Boehringer GmbH, Mannheim, Germany). Restriction endonuclease-digested DNA was eluted from 0.7% agarose gels by the freeze squeeze method (52).

**Gene disruption in *B. subtilis* ATCC 6633.** *B. subtilis* ATCC 6633 cells were transformed by the competence method (5). To obtain competent cells, 50 ml of HS medium was inoculated with a stationary overnight culture to an $A_{578}$ of 0.1. Cells were grown at 30°C until early stationary phase. Competent strains were maintained by the addition of 0.5 ml of glycerol (87%) to 5 ml of HS medium-grown cells and frozen until use as described previously (29).

For transformation, the competent aliquots were warmed up and treated as previously described (29). Subsequently, 1 μg of plasmid DNA was added, and after 2 h of agitation at 37°C, cells were plated on selective media. Circular DNA was used for transformation with replicative and integrative plasmids.

**Plasmid isolation.** The procedure of Birnboim and Doly (8) was followed with slight modification to isolate the plasmids of *E. coli*. If necessary, they were purified by use of an ultramicrocentrifuge (Beckman TL 100, rotor TLA 100.2) at 80,000 rpm for 12 h (47). Plasmid DNA from *B. subtilis* was isolated as previously described for *E. coli* (18).

**Southern hybridization.** For Southern hybridization (49), 5′-labeled oligonucleotides were used as probes at a hybridization temperature of 23°C. Oligonucleotides were labeled with [$\gamma$-$^{32}$P]ATP by using T4 polynucleotide kinase (Boehringer). Oligonucleotides and primers were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) and used without further purification. Double-stranded DNA fragments were labeled by nick translation using [$\alpha$-$^{32}$P]ATP and DNA polymerase I (Boehringer) slightly modified as described by Sambrook et al. (41).

**DNA sequencing.** Both strands of DNA were sequenced by the radioactive chain termination method (43) by using T7 DNA polymerase (Pharmacia, Freiburg, Germany) with appropriate primers.

**Polymerase chain reaction.** The polymerase chain reaction was carried out as described previously (41) in an Eppendorf MicroCycler E apparatus. By using *Taq* DNA polymerase (Boehringer), 35 cycles were performed with 20 s at 94°C, followed by 20 s at 55°C, and finally by 2.5 min at 72°C.

**Subtilin bioassay.** Test strain *M. luteus* ATCC 9341 was grown to an $A_{578}$ of 0.8, and 0.3 ml was added to 500 ml of molten Luria-Bertani agar, mixed, and poured into petri dishes (10 ml). *B. subtilis* was spread on the agar surface, and the diameter of *M. luteus* growth inhibition around the colonies was determined.

**Nucleotide sequence accession number.** The sequence data published here have been submitted to the EMBL sequence data bank under the accession number L07785.

## RESULTS

**Isolation and nucleotide sequence of *spaR* and *spaK*.** The previously isolated 4.9-kb *Xba*I fragment containing the subtilin structural gene *spaS* and genes *spaB*, *spaT*, and *spaC*, which are involved in subtilin biosynthesis (29), was used as a hybridization probe to determine adjacent restriction sites. A 7.6-kb *Hin*dIII fragment from which a 5.2-kb *Kpn*I-*Hin*dIII fragment was cloned and sequenced was identified downstream from *spaS*.

About 3 kb downstream from the subtilin structural gene *spaS*, two open reading frames could be identified. One open reading frame, *spaR*, encodes a protein of 220 amino acids and a predicted molecular mass of 25.6 kDa. The *spaR* gene is preceded by a Shine-Dalgarno sequence at an appropriate distance. A second open reading frame, *spaK*, overlaps with the 3′ end of *spaR*. The *spaK* gene probably encodes a protein of 387 amino acids with a deduced molecular mass of about 44.6 kDa. It is preceded by a putative Shine-Dalgarno sequence. Both genes show the same orientation (Fig. 1).

**Homologies of SpaR and SpaK to two-component regulators.** SpaR and SpaK may act as a two-component system consisting of an environmental sensor and a response regulator. SpaK shares homology in the C-terminal region with the conserved regions of the sensors PhoM of *E. coli* (3, 4), PhoR of *B. subtilis* (48), and NtrB of *Klebsiella pneumoniae* (31). These sensors are known to be histidine kinases which are localized in the membrane (for a review, see reference 50). As previously described for histidine kinases (50), SpaK has a conserved histidine residue at position 247 followed by a conserved asparagine residue at position 362 and a glycine-rich region between positions 370 and 380 (Fig. 2A). The histidine residue is presumably the site of autophosphorylation (50). No homologies at the protein level are present within the N-terminal region of SpaK and other sensor proteins; however, there are striking similarities between the hydrophobicity plots. Two hydrophobic regions which are probably transmembrane sequences are present at similar positions within SpaK and PhoM (Fig. 2B). These two hydrophobic transmembrane sequences possibly border a region which might be localized at the outer surface of the cytoplasmic membrane (50).

SpaR was found to share strong homologies to response regulator proteins (for a review, see reference 50). The N-terminal part of SpaR is strongly conserved, and additionally, two asparagine residues and a lysine residue are found at appropriate distances from each other. The second asparagine residue at position −51 apparently corresponds to the site of phosphorylation as shown for response regulator CheY (42). Additionally, SpaR was 35% homologous over the entire protein with response regulators OmpR of *E. coli* (12), VirG of *Agrobacterium tumefaciens* (35), and PhoB of

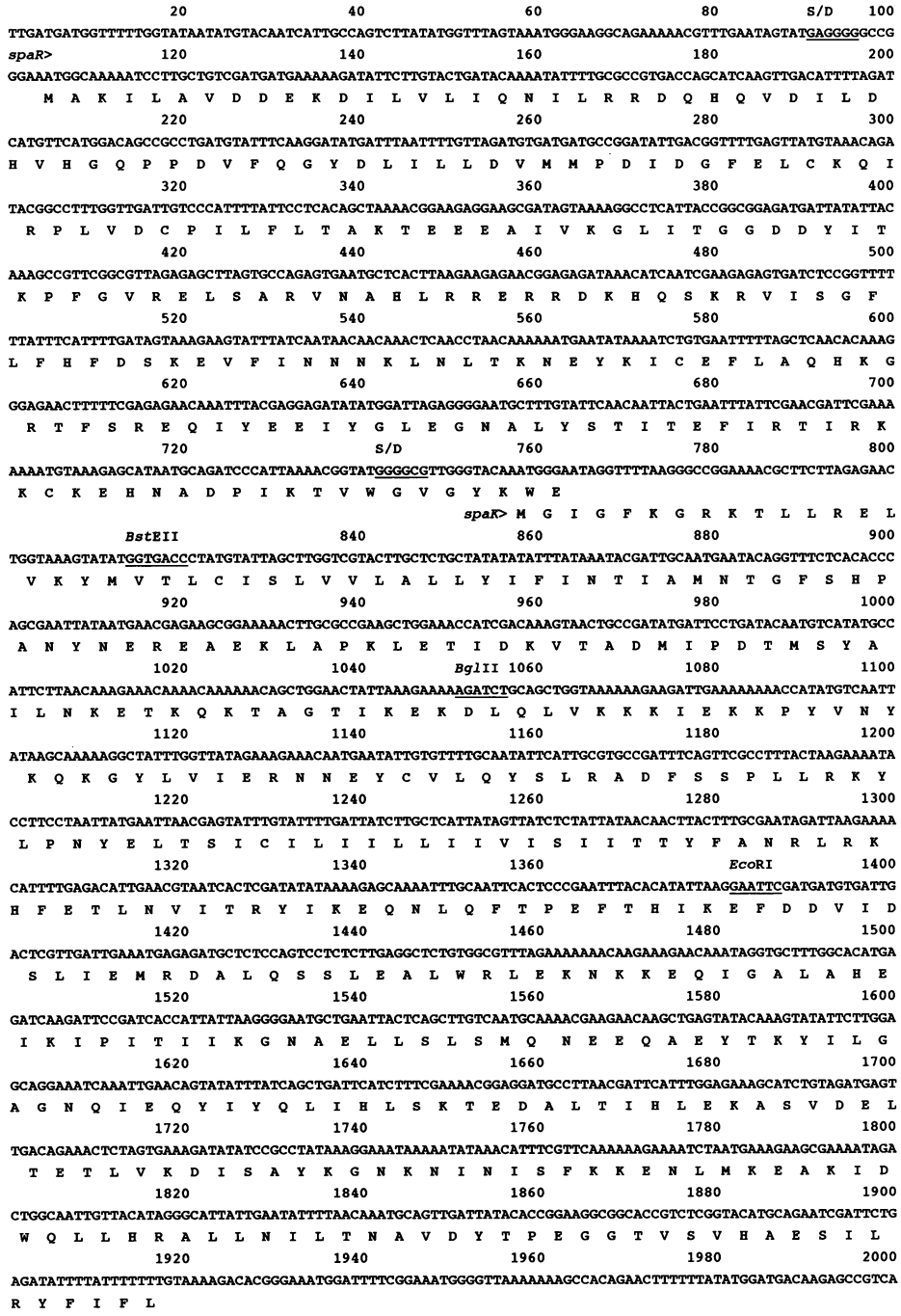


FIG. 1. Nucleotide sequences of *spaR* and *spaK*. Possible Shine-Dalgarno (S/D) sequences and restriction enzymes are underlined. Open reading frames *spaR* and *spaK* are given by the one-letter code.

*E. coli* (32) (Fig. 3). These proteins form a subfamily of response regulators (50), and the C terminus is probably responsible for DNA binding as shown by a proteolytically generated C-terminal fragment of OmpR (51). For this subfamily of response regulators, phosphorylation of the N-terminal domain is proposed to stimulate their binding activity.

**Gene deletion of *spaR* and *spaK*.** In order to establish the involvement of *spaR* and *spaK* in subtilin biosynthesis, these genes were selectively destroyed at their chromosomal loci. The *spaR* gene was amplified by polymerase chain reaction and subcloned into pUC19 (54), and finally the chloramphenicol resistance gene (*cat* gene) was inserted into the *spaR* open reading frame by using the single *Stu*I restriction site. The resulting plasmid (pCE49) was used for *spaR* gene disruption via homologous recombination (Fig. 4A). For *spaK* disruption, a 2.3-kb *Eco*RI-*Hin*dIII fragment containing the 3′ region of *spaK* was subcloned into pUC19 (54) and a 1.7-kb fragment of this construct was thereafter replaced

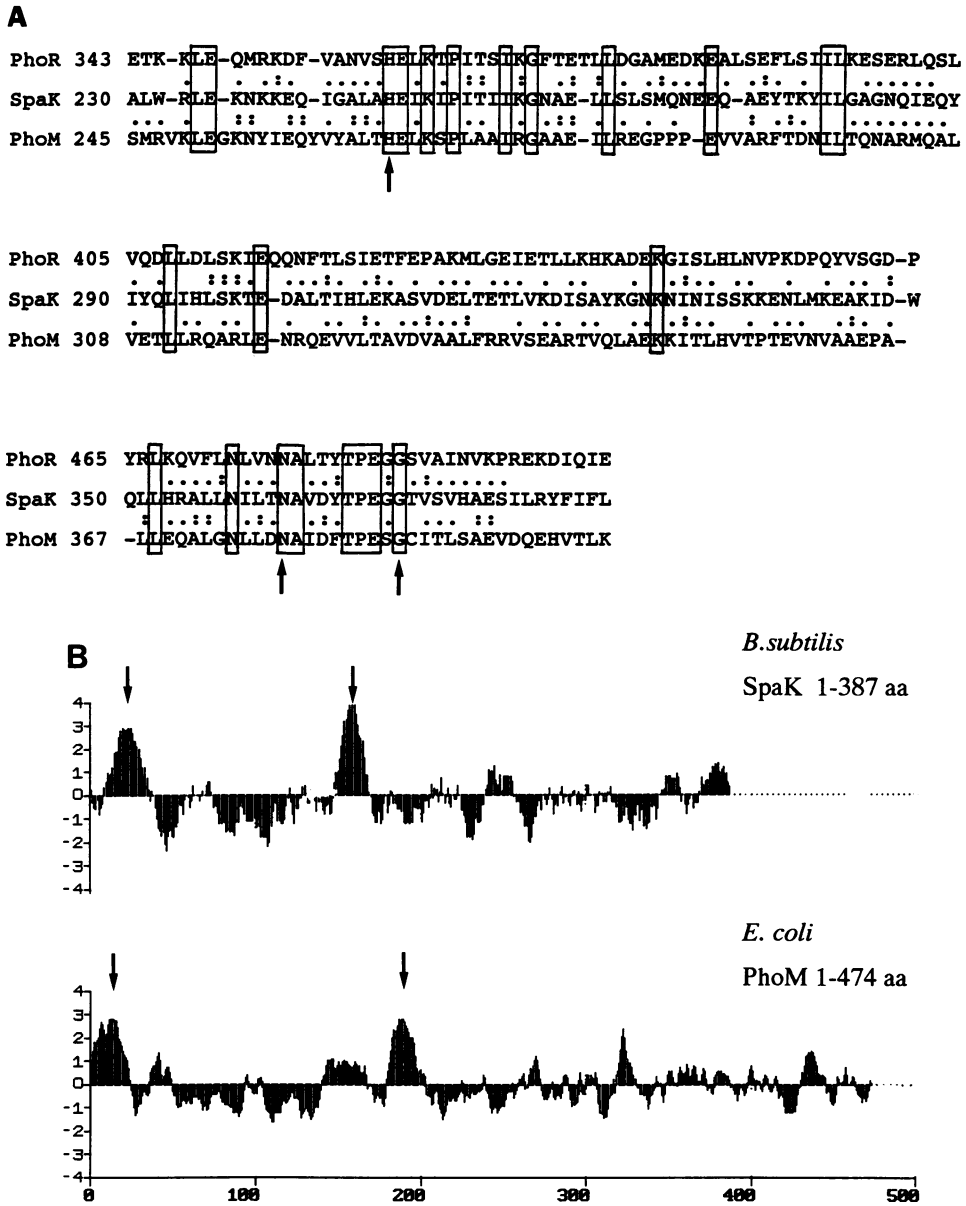


FIG. 2. Similarities between SpaK and environmental sensor proteins. (A) Sequence homologies between SpaK (*B. subtilis*), PhoR (*B. subtilis*), and PhoM (*E. coli*). Amino acid residues which are identical for all three proteins are boxed. Identical amino acid residues relative to SpaK are indicated by colons, and similar amino acid residues relative to SpaK are indicated by dots. The highly conserved histidine and asparagine residues and the glycine are indicated by arrows. (B) Hydrophobicity plots of SpaK and PhoM. The two putative membrane-spanning regions are indicated by arrows. Positive numbers refer to hydrophobicity. aa, amino acids.

by the *cat* gene, finally resulting in a 200-bp deletion of *spaK*. The resulting plasmid (pCE38) was used for gene deletion of *spaK* (Fig. 4A). About half of the obtained transformants resulted from single-reciprocal recombinations. Only double-reciprocal integrations were used for further analysis.

To analyze the correct disruption of *spaR*, the genome of the resulting transformants was verified by Southern blot analysis. The *Bam*HI-*Pvu*II fragment of plasmid pCE49 was used as a hybridization probe against chromosomal DNA digested with *Hin*dIII. A 7.6-kb signal was obtained for wild-type strain ATCC 6633, and as expected, a smaller fragment of 4.3 kb was observed for *spaR* mutants (Fig. 4B, blot 1). To confirm the correct disruption of *spaK*, an *Eco*RI-*Hin*dIII fragment of plasmid pCE38 was used as a hybridization probe. Whereas a 7.6-kb signal was obtained with DNA of wild-type cells, the *spaK* mutant revealed a signal of 1.7 kb, clearly confirming the correct replacement of the 3′ part of *spaK* by the *cat* gene (Fig. 4B, blot 2).

**Phenotype of gene disruption mutants *spaR* and *spaK*.** The gene disruption mutants *spaR* and *spaK* were tested for subtilin production by bioassay with *M. luteus* as a test organism (see Materials and Methods). Strong growth inhibition was observed with wild-type cells whereas nearly no growth inhibition occurred with *spaR* and *spaK* mutants

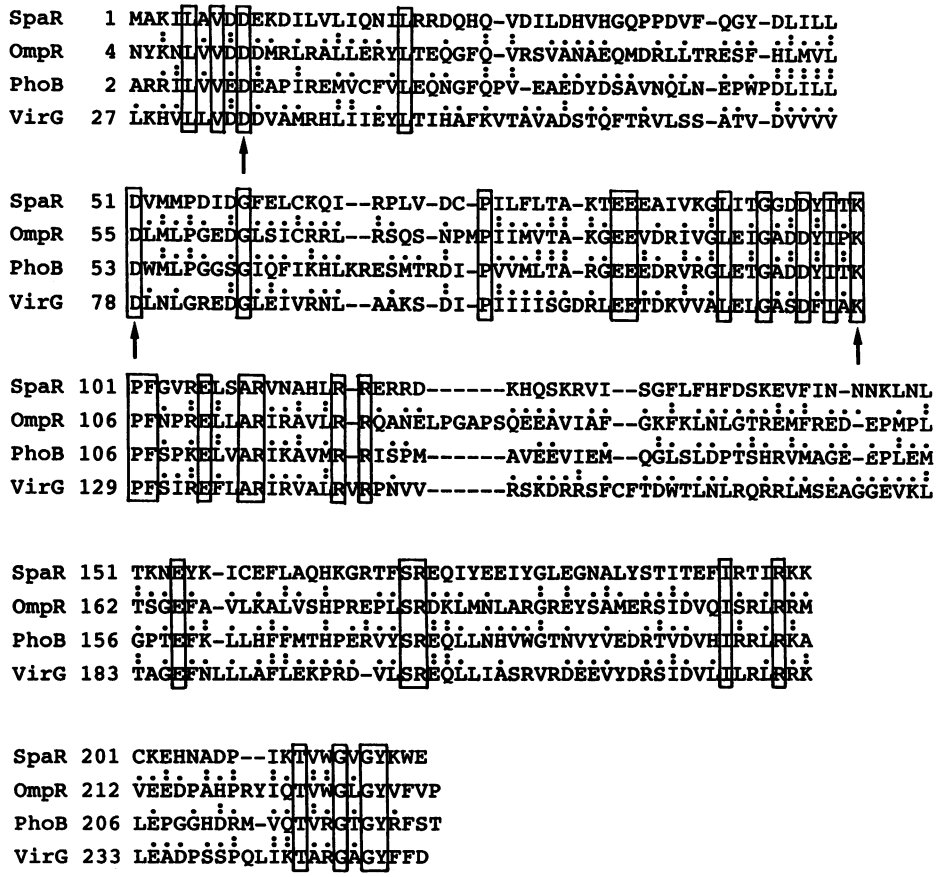


FIG. 3. Similarities between SpaR and response regulator proteins OmpR (*E. coli*), PhoB (*E. coli*), and VirG (*A. tumefaciens*). Amino acid residues which are identical for all three proteins are boxed. Identical amino acid residues relative to SpaR are indicated by colons, and similar amino acid residues relative to SpaR are indicated by dots. Highly conserved lysine and aspartate residues are indicated by arrows.

(Fig. 4C). The minor inhibition zones observed for *spaR* and *spaK* mutants result from other antibiotic activities as shown by subtilin structural gene disruptants showing similar halo sizes (29). These results established the involvement of *spaR* and *spaK* in subtilin biosynthesis.

Since *spaK* was located 5′ to *spaR*, the *spaK* gene may not have its own functional promoter. In order to exclude any polar effects of the *spaR* disruption on *spaK* transcription, the *spaR* mutant was complemented with a plasmid carrying the *spaR* gene fused to the constitutively expressed *spo* promoter (pCE40) (Fig. 5A). Transformation of *spaR* mutants with pCE40 reestablished the ability of subtilin production (Fig. 5B). This result revealed that there was no polar effect of the *spaR* disruption on *spaK* transcription and that the *spaK* gene apparently possesses its own promoter site.

## DISCUSSION

Since the isolation of lantibiotic structural genes, which proved that lantibiotics are encoded by distinct genes, several genes involved in lantibiotic biosynthesis have been identified. These open reading frames share striking similarities between the different producers as shown for epidermin (*epiB* and *epiC* [44, 45]), subtilin (*spaB*, *spaC*, and *spaT* [25, 29]), and nisin (*nisB*, *nisC*, and *nisT* [15]) (corresponding genes are indicated by the same capital letter). These similarities indicate a similar function of the respective proteins in lantibiotic maturation and transport. However, little is known about the regulation of lantibiotic biosynthesis. Nisin production strongly correlates with the presence of sucrose in the medium, and nisin is mainly produced in the early stationary growth phase (13, 14a). Subtilin production is also a regulated process. Subtilin activity and subtilin mRNAs increase in the late logarithmic growth phase (6), and the highest subtilin production was observed in the early stationary growth phase (28a).

Here we report on the identification of the regulatory elements, *spaK* and *spaR*, involved in the regulation of subtilin biosynthesis. These genes were identified adjacent to the subtilin structural gene *spaS* and the biosynthetic genes *spaB*, *spaT*, and *spaC* and possess homology to genes of a family of regulators consisting of a histidine kinase and a response regulator. Gene disruption of *spaR* and *spaK* revealed subtilin-negative mutants, which established that subtilin biosynthesis is regulated by these two genes.

All members of this family of two-component regulatory elements control responses to environmental stimuli and share protein sequence homology (50). They are usually composed of a membrane-located sensor (a histidine kinase) which activates a response regulator acting at the level of transcription. Signal transduction occurs via phosphorylation of a histidine residue in the histidine kinase by ATP and is followed by the transfer of the phosphoryl group to an aspartic acid residue in the response regulator. These sensor

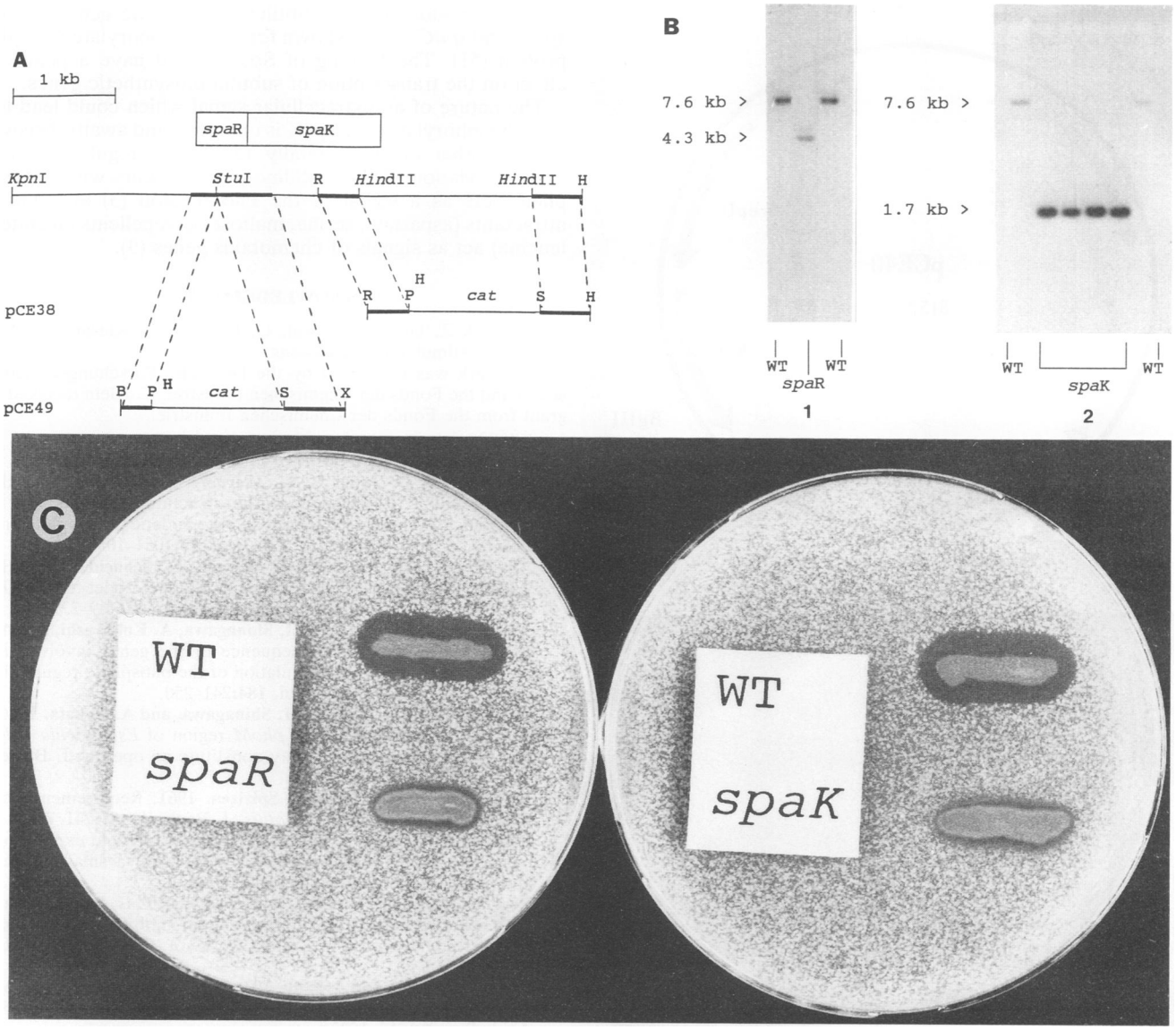


FIG. 4. Gene disruption of *spaR* and *spaK*. (A) Plasmids pCE49 and pCE38 constructed for gene disruption of *spaR* and *spaK*. The genomic open reading frames are boxed. The regions where homologous recombinations occurred are indicated by dashed lines. Solid bars correspond to homologous regions between chromosomal DNA and the plasmids. B, *Bam*HI; P, *Pvu*II; R, *Eco*RI; S, *Sma*I; X, *Xba*I; H, *Hin*dIII. (B) Southern blot analysis of *B. subtilis* wild type (WT) and mutant DNA. Chromosomal DNA was digested with *Hin*dIII. The 7.6-kb fragment found for wild-type lanes is the one we cloned from chromosomal DNA. Probes used are mentioned in the text. The fragments we detected in mutant strains have the expected size, as can be deduced from Fig. 4A. (C) Bioassay for subtilin production. The wild type (WT) and gene disruption mutants *spaR* and *spaK* were streaked onto plates containing *M. luteus* as a test organism (see Materials and Methods).

proteins share a conserved sequence in their C-terminal domains. Almost all of these histidine kinases have a conserved histidine which precedes a conserved asparagine by about 100 residues. In their N-terminal regions, they possess two membrane-spanning sequences.

The response regulators, on the other hand, are cytoplasmic and are known to bind to DNA (37, 38). They are homologous in their N-terminal domain of 100 amino acids, including two aspartate residues and one lysine residue at similar distances from each other. For epidermin biosynthesis, we also identified a regulatory protein, *epiQ*, near the structural gene *epiA*. In contrast to most other response regulators, *epiQ* is only similar to the supposed DNA-binding C terminus of *spaR* (44, 45). A gene homologous to histidine kinases has not been found, also indicating that this regulation is different from that of the two-component regulatory system described for the regulation of subtilin biosynthesis.

Two-component environmentally responsive regulator systems have been reported for both gram-positive and gram-negative bacteria, and they are involved in chemotaxis, osmoregulation, nitrogen and phosphate regulation, anaerobiosis, and sporulation. SpaK, which shares homology with the prominent histidine kinases PhoR, NtrB, and PhoM, has two hydrophobic putative membrane-spanning sequences in its N terminus with a hydrophilic region in

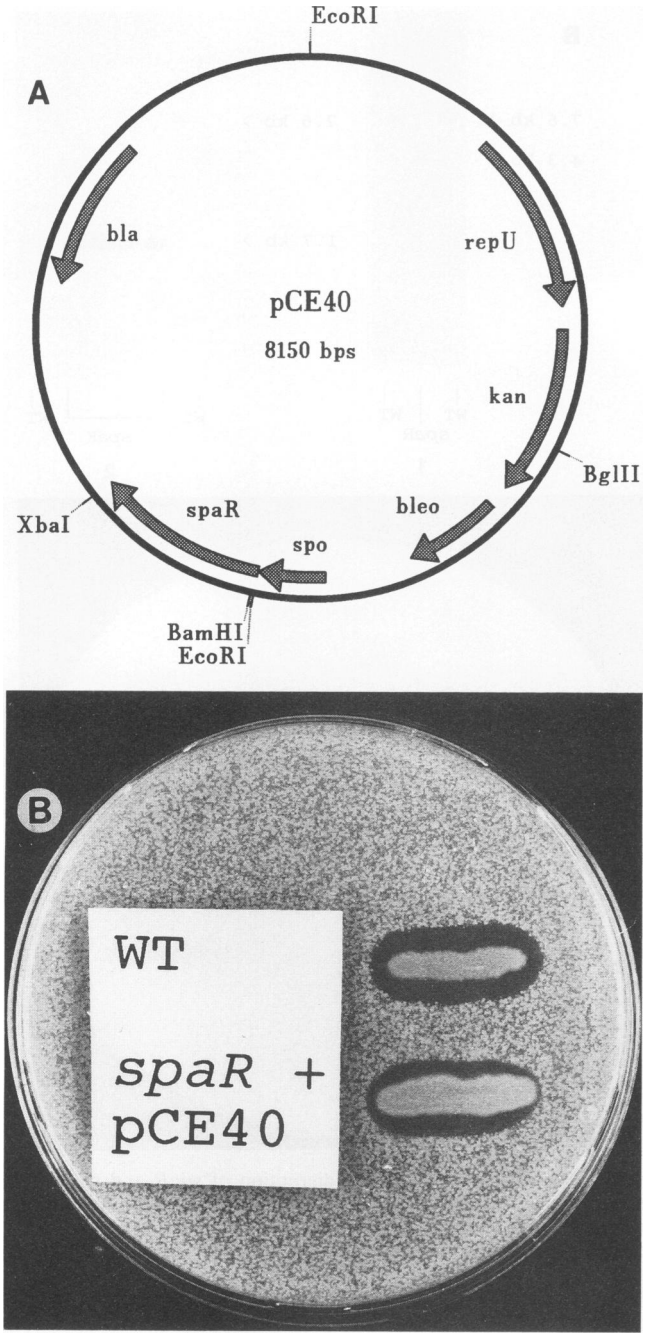


FIG. 5. Complementation of the *spaR* disruption mutant. (A) Plasmid pCE40 was used for complementation of *spaR*. (B) Bioassay for subtilin production. The wild type (WT) and the complemented *spaR* disruption mutant (*spaR* + pCE40) were tested with *M. luteus* as a test organism.

between. This indicates that SpaK may be located in the membrane. SpaK may function by transducing an extracellular signal within the hydrophilic part of the membrane-spanning helices by autophosphorylation of the His-247 in its conserved N-terminal region. The phosphorylated SpaK protein would now be able to transfer the phosphoryl group to the Asp-51 residue of the SpaR protein. The phosphorylated SpaR protein may thereafter bind to sequences in the promoter regions of the subtilin biosynthetic genes *spaB*, *spaT*, and *spaC* as was shown for the phosphorylated OmpR protein (51). The binding of SpaR would have a positive effect on the transcription of subtilin biosynthetic genes.

The nature of an extracellular signal which could lead to autophosphorylation of SpaK is unknown and awaits discovery. In other environmentally responsive regulators, the phosphorylation of the histidine kinase occurs when phosphate acts as a signal of the Pho regulon (3) and when attractants (aspartate, serine, maltose) or repellents (acetate, leucine) act as signals of chemotaxis genes (9).

## ACKNOWLEDGMENTS

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