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Gramicidin S Biosynthesis Operon Containing the Structural Genes *grsA* and *grsB* Has an Open Reading Frame Encoding a Protein Homologous to Fatty Acid Thioesterases

JÖRN KRÄTZSCHMAR, MICHAEL KRAUSE, AND MOHAMED A. MARAHIEL*

Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, Franklinstrasse 29, D-1000 Berlin 10, Federal Republic of Germany

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The DNA sequence of about 5.9 kilobase pairs (kbp) of the gramicidin S biosynthesis operon (*grs*) was determined. Three open reading frames were identified; the corresponding genes, called *grsT*, *grsA*, and *grsB*, were found to be organized in one transcriptional unit, not two as previously reported (M. Krause and M. A. Marahiel, J. Bacteriol. 170:4669–4674, 1988). The entire nucleotide sequence of *grsA*, coding for the 126.663-kilodalton gramicidin S synthetase 1, *grsT*, encoding a 29.191-kilodalton protein of unknown function, and 732 bp of the 5′ end of *grsB*, encoding the gramicidin S synthetase 2, were determined. A single initiation site of transcription 81 bp upstream of the *grsT* initiation codon GTG was identified by high-resolution S1 mapping studies. The sequence of the *grsA* gene product showed a high degree of homology to the tyrocidine synthetase 1 (TycA protein), and that of *grsT* exhibited a significant degree of homology to vertebrate fatty acid thioesterases.

In response to certain nutrient conditions usually associated with nutrient depletion, gram-positive bacteria of the genus *Bacillus* induce the process of endospore formation as well as the production of secondary metabolites such as peptide antibiotics and extracellular proteases (4, 26, 41). In both cases, genes are activated at the transition from logarithmic to stationary phase of growth. Expression of these genes depends on the products of *spo0* loci (10, 26, 30, 41). To understand the complex relationship between induction of sporulation and production of secondary metabolites, we and others have isolated and studied the organization and regulation of some antibiotic biosynthesis genes at the molecular level. Genes encoding multifunctional enzymes involved in biosynthesis of the cyclic antibiotics bacitracin, gramicidin S, and tyrocidine have been identified (16, 20, 33). The nonribosomal synthesis of these antibiotics by the so-called protein-thiotemplate mechanism has been studied extensively (18, 22, 23, 25). In analogy with fatty acid synthetase, the cofactor 4′-phosphopantetheine is involved in translocation of the growing polypeptide chain through transthiolation. However, there is one important difference: the fatty acid chain is synthesized by condensation of identical carbon units, whereas in polypeptide synthesis, different amino acids are polymerized in a defined sequence given by the position of the corresponding domain on the multienzyme.

Recently, we reported that the biosynthesis genes for gramicidin S, *grsA* and *grsB*, and those involved in tyrocidine biosynthesis, *tycA* and *tycB*, are clustered (20, 33). We demonstrated that *tycA* and *tycB* are organized in an operon and presented evidence for their expression as a polycistronic transcriptional unit (33). In this paper, we present the DNA sequence of a major part of the gramicidin S gene cluster. Sequence data revealed the presence of an open reading frame (ORF), called *grsT*, encoding a protein similar to the fatty acid thioesterases isolated from rat mammary gland and duck uropygial gland (38, 39). In addition, S1 nuclease protection studies located a single transcription initiation site upstream of the *grsT* ORF, which may control the expression of the entire antibiotic biosynthesis operon in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* JM105 and JM83 (32) were used as hosts for the recombinant plasmids and were grown in L-broth medium supplemented with the appropriate antibiotics. *Bacillus brevis* ATCC 9999, the gramicidin S producer strain, was grown in nutrient broth (28) or sporulation medium (14).

Plasmid construction and DNA sequencing. DNA fragments obtained after partial or complete digestion of the previously described plasmids pGS1-2, pMK12, pMK21, and pKE1 (20) with restriction enzymes were subcloned into plasmid vectors (pUC18, pUC19, and pGEM-3Z; Promega Biotec) or the replicative forms of the bacteriophage vectors M13mp18 and -mp19 (32). Fragment preparation and ligation were achieved in low-melting-point agarose (Bethesda Research Laboratories, Inc.) as described by Crouse et al. (7).

Plasmids were prepared from *E. coli* cells by the alkaline extraction method of Birnboim and Doly (3). Single-stranded DNA of M13 derivatives was isolated by the method of Messing (32).

Sequencing of single-stranded DNA and alkali-denaturated plasmid DNA by the dideoxy-chain termination method of Sanger et al. (40), using modified T4 DNA polymerase (Sequenase; United States Biochemical Corp.), yielded a set of overlapping partial sequences from both DNA strands of the *grs* operon region (Fig. 1). For the S1 mapping experiments, DNA fragments labeled at one 5′ end were sequenced by the chemical cleavage method of Maxam and Gilbert (31).

Sequence analysis was carried out by using the programs WORDSEARCH, BESTFIT, and GAP, included in the University of Wisconsin Genetics Computer Group sequence analysis software package.

RNA preparation. Cells of *B. brevis* ATCC 9999 were

* Corresponding author.

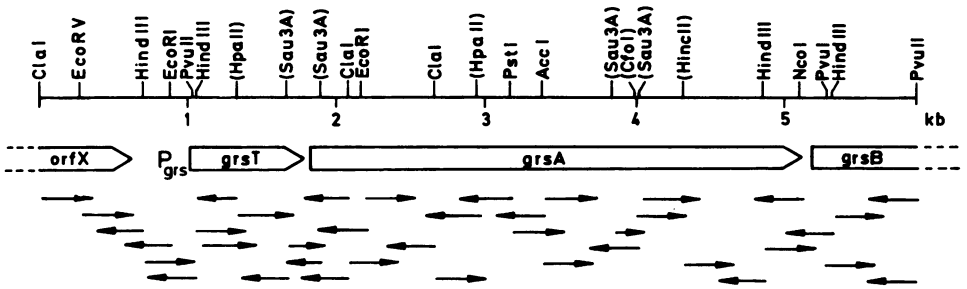

FIG. 1. Partial restriction map and sequencing strategy for the 5′ region of the *grs* operon. Potential ORFs, direction of transcription, and relevant restriction sites used for sequencing are indicated; for restriction enzymes in parentheses, not all sites are shown. Arrows indicate the direction of sequencing and the extent of sequence obtained from individual clones, as determined by the dideoxy-chain termination procedure.

grown in Hanson sporulation medium (14) at 37°C. Samples (50 ml) of cell culture were collected at different times around T_0, the production phase of gramicidin S. Total cellular RNA was prepared by the procedure of Penn et al. (37) as modified by Igo and Losick (15). RNA pellets were suspended in 200 μl of diethylpyrocarbonate-treated water containing 10 U of RNase inhibitor (Boehringer GmbH) per ml.

S1 nuclease protection assay. Mapping of 5′ ends of mRNA was performed by the method of Murray (35). Double-stranded DNA probes were 5′ end labeled by a standard method (27).

Mixtures of DNA probes (ca. 10 ng; specific activity, 10 μCi/μg) and total cellular RNA (20 μg) were dried in a vacuum microfuge and suspended in 20 μl of hybridization buffer [3 M sodium trichloroacetate, 50 mM piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 5 mM EDTA]. After heating at 70°C for 5 min, samples were incubated at 45°C for 4 h. Reassociation was terminated by addition of 200 μl of ice-cold solution containing 250 mM NaCl, 40 mM sodium acetate (pH 5.5), 1 mM $ZnCl_2$, 20 μg of denatured calf thymus DNA per ml, and 2,000 U of S1 nuclease (Boehringer) per ml. After 30 min at 37°C, samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, denaturated in 10 μl of formamide loading dye, and electrophoresed on 6% polyacrylamide–urea gels (31). Dried gels were exposed to Kodak XAR5 film for 16 h at room temperature.

RESULTS

Nucleotide sequence of the *grs* operon. In a previous work, we reported the identification and physical characterization of a 14-kilobase-pair (kbp) DNA fragment from an EMBL3 library containing the entire *grsA* gene and a major part of the *grsB* gene (20). Approximately 5.9 kbp of this region, located between the far-left *Cla*I site and the second-left *Pvu*II site, were sequenced (Fig. 1). The sequence showed four potential ORFs (Fig. 2). *orfX* extends from the 3′-end 678 nucleotides to the end of the determined sequence. The 5′ end of the *grsT* coding region is separated by a 326-bp noncoding sequence from the 3′ end of *orfX*. The *grsT* gene encodes a protein of 256 amino acid residues with a deduced molecular mass of 29,191 daltons (Da). It is followed by 26 untranslated nucleotides and then by 3,297 nucleotides of the *grsA* coding region. *grsA* encodes the 1,089-amino-acid (126,661 Da) gramicidin S synthetase 1, as indicated previously by immunological and enzymatic studies (20). The deduced amino acid sequence of the *grsA* gene has recently been confirmed by determination of the amino-terminal sequence (10 residues) of the purified gramicidin S synthetase 1 from *B. brevis* (J. Vater, W. Schlumbohm, J. Salnikow, K. D. Irrgang, M. Miklus, T. Choli, and H. Kleinkauf, Biol. Chem. Hoppe-Seyler, in press). The *grsA* gene is followed by a noncoding region of 71 nucleotides and then by an ORF (*grsB* gene) ascribed to the multifunctional enzyme gramicidin S synthetase 2 (see below). About 730 nucleotides of coding region of the *grsB* gene were sequenced (Fig. 2). *grsA* and *grsB* reading frames are initiated at an ATG codon preceded by reasonable ribosome-binding sites. In contrast, *grsT* is initiated at a GTG codon that is preceded by two overlapping strong ribosome-binding sites, one of them located at optimal distance. The unusual GTG initiation codon has been reported for the *Bacillus* genes *spoVG*, *0.3 kb* (34), *sprE* (47), *cerB* (12).

The close location of the *grsB* gene to the 3′ end of *grsA* was confirmed by deletion analysis at the *grsB* 3′ end and by the expression of the corresponding truncated but immunoreactive gene products (J. Krätzschmar, Diplomarbeit, Technische Universität Berlin, 1989). For example, the previously constructed (20) plasmids pKC2, pMK18, and pKE1, containing *grsB* inserts of 2.7, 3.3, and 4.4 kbp (seen from the 3′ end of the *grsB* moiety to its initiation codon located at position 5150) were shown to express immunoreactive GrsB fragments of 105, 135, and 170 kDa, respectively (data not shown).

High-resolution S1 protection analysis of the *grs* promoter region. Analysis of the DNA sequence in the intergenic regions between *grsT* and *grsA* (26 bp) as well as between *grsA* and *grsB* (71 bp) revealed no sequences resembling the RNA polymerase-binding sites utilized by any of the holoenzyme forms so far characterized in the genus *Bacillus* (26). S1 protection experiments (data not shown) using probes from the two intergenic regions (the 1,042-bp *Pvu*II-*Cla*I fragment for the *grsT-grsA* region and the 447-bp *Hin*dIII fragment for the *grsA-grsB* region) showed full protection and defined no sites of transcription initiation. These results indicate that the promoter for the *grs* operon may be located in front of the *grsT* gene. To determine the location of the transcriptional start site(s) in this region, an S1 protection experiment was carried out by using the 5′-end-labeled 335-bp *Hin*dIII fragment spanning the area from nucleotide 32 downstream of the 3′ end of *orfX* to nucleotide 65, within the *grsT* coding sequence (Fig. 2). The results obtained by using total RNA isolated from *B. brevis* cells at T_0 are shown in Fig. 3. The protected fragments were run against a Maxam-Gilbert sequencing ladder, with the result that a single prominent band within a much weaker band set was found to correspond to the adenine residue indicated in Fig.

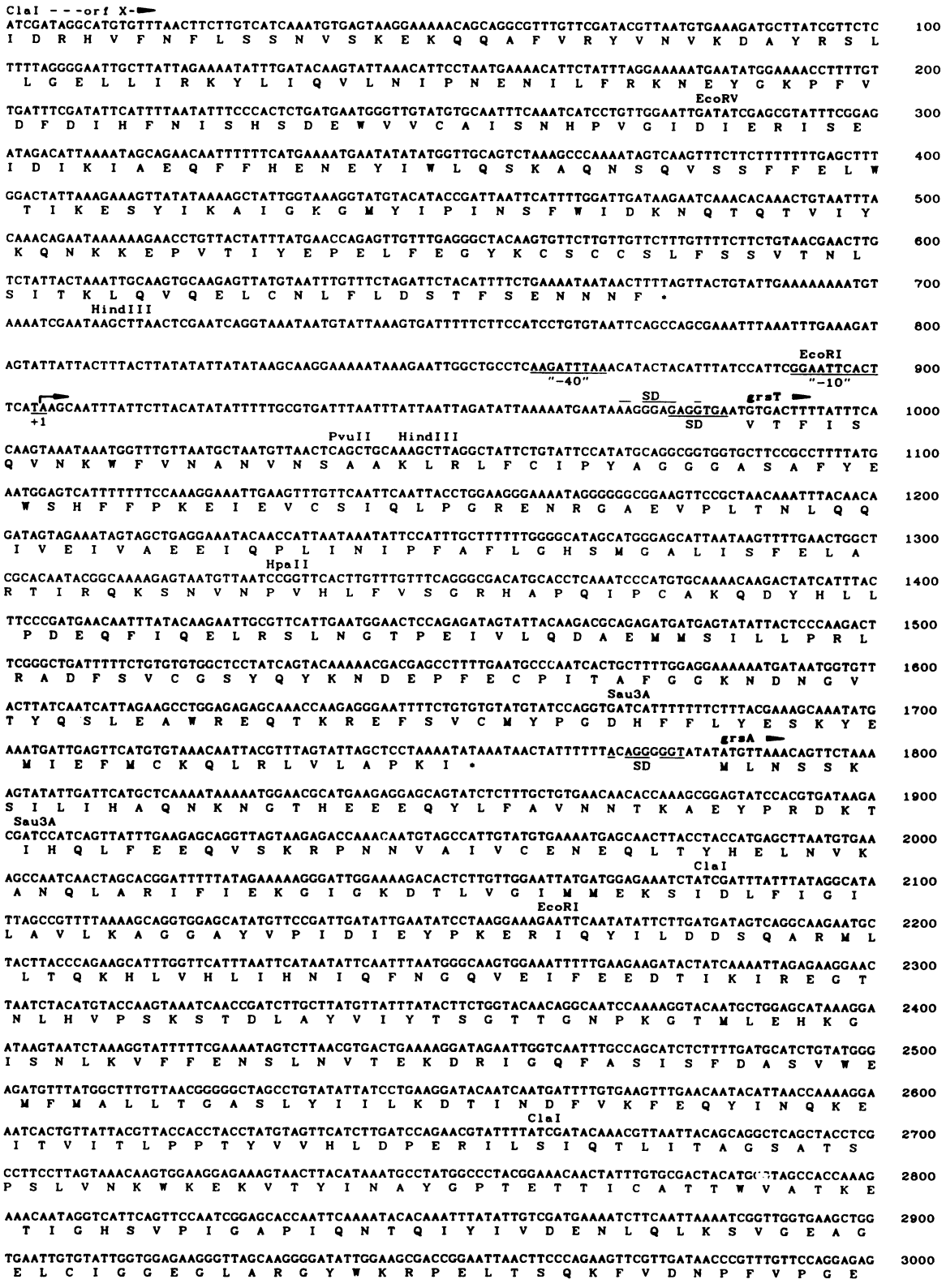

FIG. 2. DNA sequence of the 5,881-bp DNA fragment shown in Fig. 1. The encoded amino acid sequences of four ORFs (3′ end of *orfX*; *grsT*, *grsA*, and the 5′ end of *grsB*) are shown. Potential ribosome-binding sites (SD) and the putative promoter (−10 and −40) region are underlined. The long arrow at position +1 upstream of *grsT* coding region defines the transcription initiation site determined by high-resolution S1 mapping.

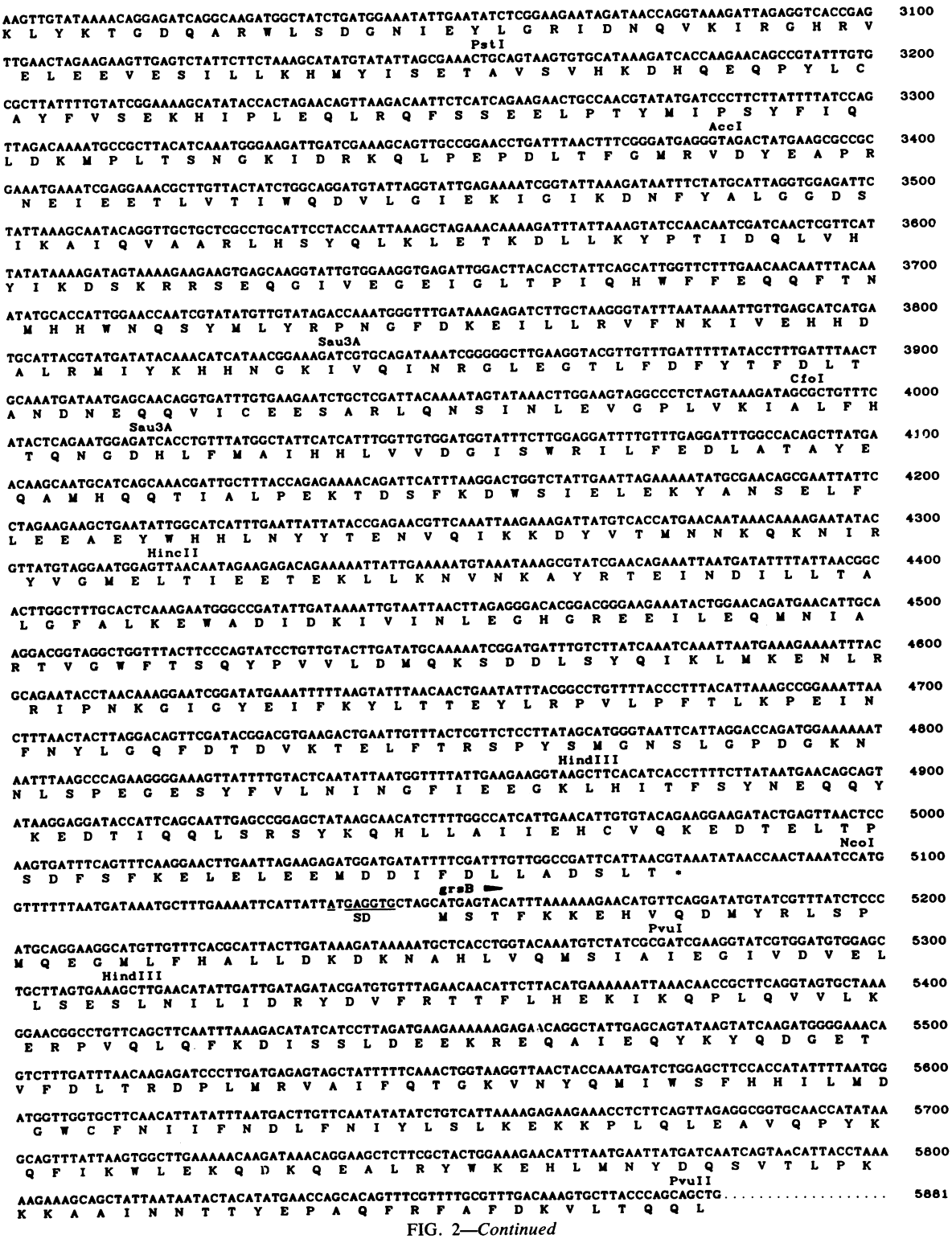

FIG. 2—*Continued*

3. This experiment located the major start site of transcription initiation for the *grs* operon exactly 81 bp (position +1) upstream of the *grsT* initiation codon GTG. Table 1 compares the DNA sequence found upstream of the identified transcription initiation site with the recognition sequences of three well-characterized *Bacillus subtilis* promoters (1, 5, 6, 15, 17, 47).

Comparison of the GrsA amino acid sequence with that of TycA and the amino termini of GrsB and TycB. Biosynthesis of the related peptide antibiotics gramicidin S and tyrocidine by two different *B. brevis* strains is initiated by the enzymes gramicidin S synthetase 1 (GrsA) and tyrocidine synthetase 1 (TycA), respectively (21). Both enzymes activate and racemize the amino acid phenylalanine as the first step in the

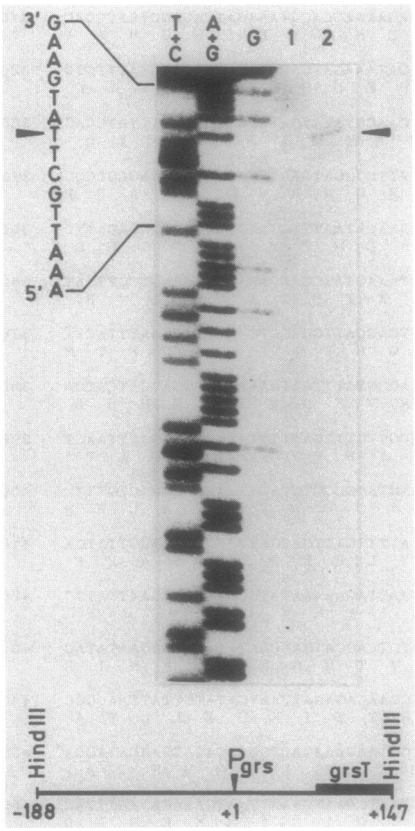

FIG. 3. High-resolution S1 nuclease mapping of the *grs* transcript. The radiolabeled DNA probe (a 335-bp *Hin*dIII fragment) shown at the bottom was combined with total RNA of *B. brevis* isolated at T_0 under hybridization conditions (see Materials and Methods). After S1 treatment, the nuclease-resistant hybrids were denaturated and resolved by electrophoresis on a 6% polyacrylamide–8 M urea gel. The probe was also subjected to the base-specific cleavage reactions of Maxam and Gilbert (31). Arrowheads indicate the position of a protected band (lane 2). Lane 1, S1-treated probe without RNA. The *grs* promoter is indicated by Pgrs at the position of transcription initiation (+1).

initiation of the corresponding peptide synthesis. The activated amino acid is transferred to a thiol group (a cysteine residue) on the enzyme, yielding a covalently bound thioester-linked amino acid (25). The two enzymes have similar molecular masses and cross-react immunochemically (29). To evaluate the possibility that the two enzymes might have a common evolutionary ancestry or have been generated by gene duplication, we compared their predicted amino acid sequences. Comparison of the deduced amino acid sequences of GrsA and TycA (46) revealed an extensive degree of homology of over 56% (Fig. 4A). If similar amino acids are also considered, the degree of homology increases to over 70%. Although the TycA protein is 11 amino acids shorter and the lowest degree of homology is located at the amino termini, no extensive deletions or insertions in the internal segments have been observed. It is important to note that 4 of 6 cysteine residues in GrsA (residues 331, 377, 474, and 1065) share almost the same location with 4 of 12 cysteine residues in TycA.

We also compared the available sequences of the amino-terminal parts of GrsB and TycB (Fig. 4B). The *grsB* gene encodes the gramicidin S synthetase 2 (280 kDa), a multifunctional enzyme able to activate and polymerize the amino acids proline, valine, ornithine, and leucine (11). The *tycB* gene encodes the tyrocidine synthetase 2 (230 kDa), which activates and polymerizes the amino acids proline, phenylalanine, and D-phenylalanine (23). The comparison revealed over 54% identical residues within the first 244 residues of both enzymes.

Similarity of GrsT to other proteins. A computer search for similarities between the deduced amino acid sequence of the GrsT protein and other protein sequences revealed a significant degree of homology to the medium-chain *S*-acyl fatty acid synthetase thioester hydrolase (thioesterase II) from rat mammary gland (39) and to another thioesterase II from mallard duck uropygial gland (38) (Fig. 5). The overall level of homology was more than 30%. Both thioesterases and the GrsT protein have a molecular mass of approximately 29 kDa. Both thioesterases are serine active-site enzymes that hydrolytically release the fatty acid acyl moiety from its thioester linkage to the 4′-phosphopantetheine prosthetic group (44). The rat mammary gland thioesterase II can functionally replace the thioesterase domain of the fatty acid synthetase (thioesterase I) but releases shorter fatty acid chains (24). The sequence surrounding the proposed active-site serine in both thioesterases, GHSFG, was found to be almost perfectly conserved in the GrsT protein (GHSMG; positions 99 to 103) (Fig. 5). In addition, two regions of striking homology in both thioesterases, residues 28 to 38 and 235 to 246, were found to be strongly conserved in the GrsT protein.

DISCUSSION

We have determined the nucleotide sequence of a major part of the gramicidin S biosynthesis operon and located a transcription initiation site through high-resolution S1 protection mapping. The results revealed the presence of three ORFs, referred to as *grsT*, *grsA*, and *grsB*, and indicated that transcription was initiated 81 bp upstream of the *grsT* gene and extended through *grsA* and *grsB*. The entire nucleotide sequences of *grsT* (771 bp) and *grsA* (3,297 bp) and about 730 bp of the 5′-end coding region of *grsB* were determined. Two short noncoding intergenic regions of 26 and 71 bp were observed between *grsT* and *grsA* and between *grsA* and *grsB*, respectively. No sites for initiation

TABLE 1. Comparison of the putative *grs* promoter sequence with recognition sequences of minor sigma factor promoters from *B. subtilis*[a]

Promoter	−35 region	Spacer	−10 region
grs	TCAAGATTTAAA	CATACTACATTTATCCATTC	GGAATTCACT
ctc	AGG.TTTAAA	TCCTTATCGTTATG	GGTATTGTTT
spoVG (P1)	GCAGGATTTCAG	AAAAAATCGT	GGAATTGATA
rpoD (P3)	GCAGGAGTTTAA	TGGAGGGATGG	AGAATTACTC

[a] DNA sequences are from references 5, 6, 15, and 17.

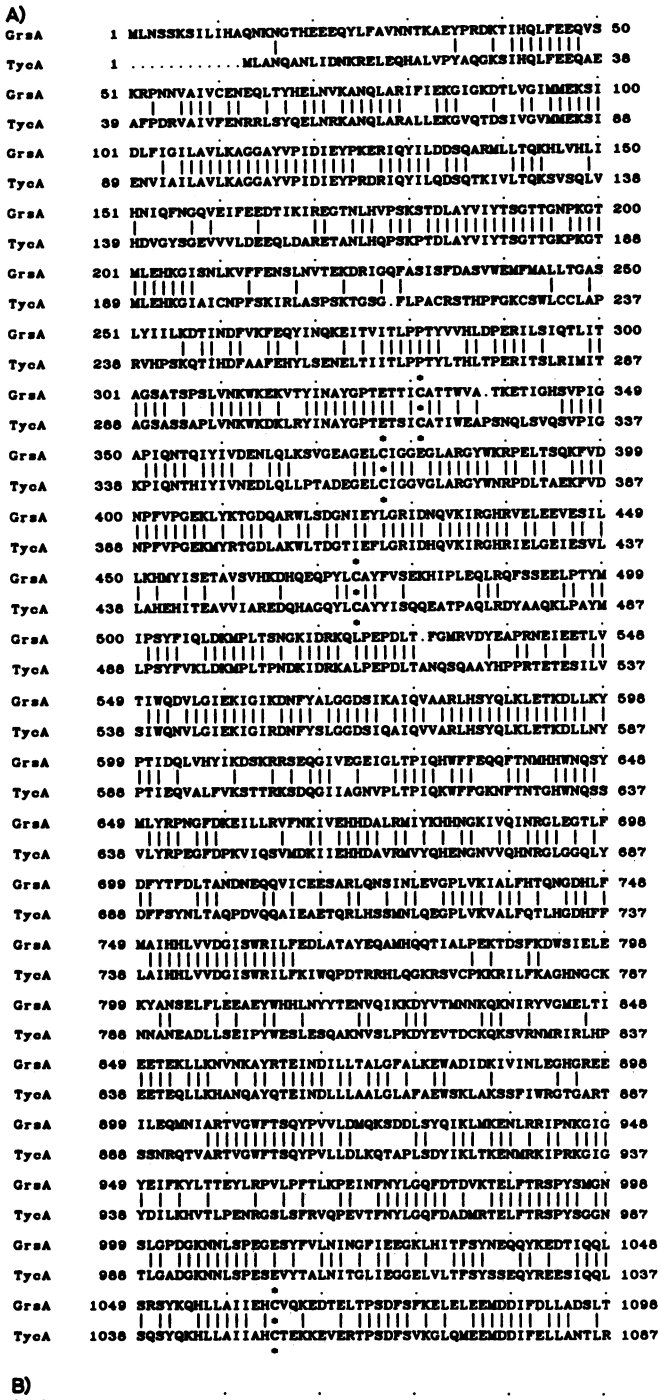

FIG. 4. Alignment of the deduced amino acid sequences of *grsA* and *tycA* (A) and of the amino-terminal sequences (about 244 residues) of *grsB* and *tycB* (B). Matches are marked by vertical lines between the corresponding amino acids; asterisks indicate positions of conserved cysteine residues. The sequences of *tycA* and *tycB* are from references 46 and 33, respectively.

of transcription were found in these two regions, and analysis of their sequences revealed no potential sites for RNA polymerase binding. This result suggests that transcription is directed only by the putative promoter mapped upstream of the *grsT* gene. The sequence data, in addition to the results obtained from S1 protection analysis and from deletion studies at the 3′ end of the *grsB* gene (J. Krätzschmar, Diplomarbeit), corrected our earlier observations on the location of *grsB* and on the direction of *grsA* transcription (20). In conclusion, these data strongly suggest that the genes *grsT*, *grsA*, and *grsB* are organized in an operon and transcribed undirectionally as one polycistronic transcriptional unit.

The DNA sequence comparison in Table 1 reveals a certain degree of homology between the putative *grs* promoter sequence and *B. subtilis* promoters recognized by minor forms of RNA polymerase sigma factors: both *ctc* and *spoVG* are transcribed by σ^B (σ^{37}) RNA polymerase holoenzyme in vitro (15, 17). Whereas *ctc* transcription is blocked in *sigB* null mutant strains, recent data suggest that the *spoVG* promoter, like one of three *rpoD* operon promoters, is also recognized by σ^H (σ^{30}) RNA polymerase holoenzyme (5, 6). The spacing of the putative −10 and −35 sequences of the *grs* promoter is 6 to 10 bp higher than in the known σ^B and σ^H promoters. Recognition sequences spaced by 20 bp are not expected to form a functional complex with RNA polymerase holoenzymes (34). However, spacing of 21 and 22 bp was observed in two functional promoters, the developmentally regulated promoters of *spoIIE* and *spoIIG* (13, 19). In contrast to the putative *grs* promoter, those of *spoIIE* and *spoIIG* are known to be recognized by the vegetative σ^A (σ^{43})-associated form of RNA polymerase. To overcome the inappropriate spacing between the consensus sequences, an interaction with modified conventional forms of RNA polymerase or the assistance of a helix-distorting protein in transcription has been suggested (13). Perhaps new, uncharacterized forms of RNA polymerase holoenzymes might recognize these unusually spaced promoters. We regard it as an open question whether the *grs* promoter is actually utilized in vivo by RNA polymerase holoenzyme containing minor sigma factors. This evaluation should await studies on the transcription in *B. subtilis* strains bearing a *sigB* null mutation (2).

The *grsA* gene encodes a protein of 126,631 Da, which is in agreement with the apparent molecular mass of 120,000 Da previously determined for the gramicidin S synthetase 1 (29, 45). The DNA sequence is further supported by the coincidence of its predicted amino acid sequence with the recently determined amino-terminal sequence (10 residues) of the gramicidin S synthetase 1 purified from *B. brevis* (Vater et al., in press). Comparison of the GrsA sequence with the predicted amino acid sequence of the *tycA* gene product revealed over 56% of identical residues. These results supported the functional relationship, since both enzymes activate and racemize the amino acid phenylalanine as the first step in nonribosomal biosynthesis of the peptide antibiotics gramicidin S and tyrocidine. On the basis of these similarities, four of six cysteine residues in GrsA were found to be conserved in TycA, suggesting a possible role for one of

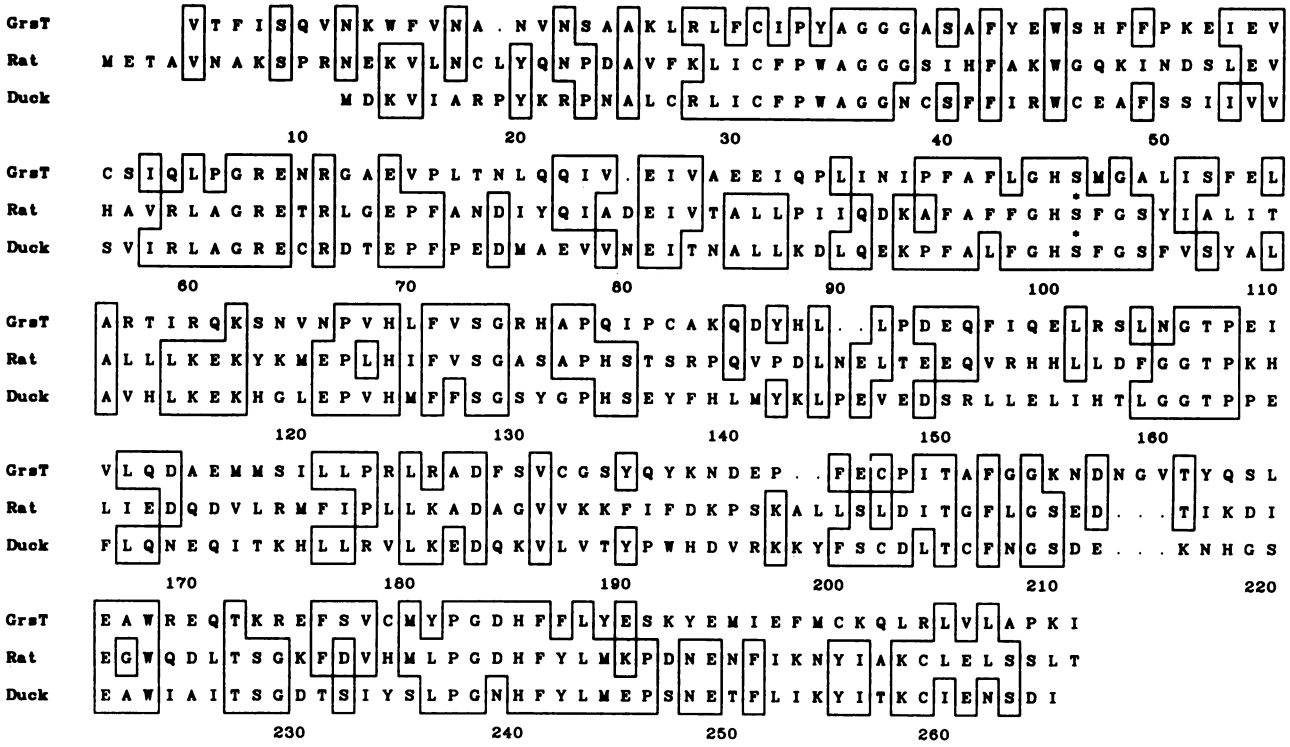

FIG. 5. Comparison of the amino acid sequence encoded by *grsT* (GrsT) with the amino acid sequences of rat mammary gland (rat) and mallard duck uropygial gland (duck) thioesterase II enzymes. The sequences have bene aligned via the proposed active-site region (GHSXG). Homologous residues are enclosed in boxes; the active-site serine is indicated by asterisks.

them in thioester formation. This hypothesis can now be confirmed by deleting or modifying these residues. The observed homology in the amino-terminal regions of GrsB and TycB sequences deduced from their nucleotide sequences suggest that this conserved region (at least 244 residues) might be part of the proline activation domain. Proline is the first amino acid activated by both enzymes. A cysteine residue (position 154 in GrsA; position 155 in TycB) that might be the site of proline thioester formation is conserved in both sequences. Completion of the nucleotide sequences for *grsB* and *tycB*, which is in progress, may provide further insight into the domain structures of these proteins.

One of the most surprising features of the sequence analysis presented here was the discovery of the striking homology between the *grsT* gene product (GrsT) and fatty acid thioesterase II enzymes. The *grsT* gene encodes a polypeptide of 256 residues with a calculated M_r of 29,191, which is very close to those of rat (molecular weight, 29,471) and mallard duck thioesterase II (M_r, 28,800) enzymes (38, 39). The overall level of homology is about 30%. Interestingly, the active-site serine residue that is a part of the motif GXSXG in thioesterase II was found to be conserved within the same motif in GrsT. This active-site motif is also present in the sequence of the thioesterase domain of fatty acid synthetase from rat (thioesterase I) (36, 43). In addition, two other clusters of conserved homologies in thioesterase II enzymes located near the N and C termini were also found to be conserved in the GrsT sequence (Fig. 5). It is now well established that thioesterase II enzymes form complexes, in some specialized tissues, with their respective fatty acid synthetases, causing the premature release of medium-chain-length (C_8, C_{10}, and C_{12}) fatty acids (24). In contrast, nothing at all is known about a possible function for the GrsT protein. Interestingly, the active-site GHSXG motif also occurs in lipases (8) and in fatty acid synthetase domains associated with acyl group transfer (malonyl/palmitoyl transferase and acetyl transferase) (9, 42). During gramicidin S biosynthesis, repeated acyl transfer reactions of the growing peptide chain take place between thiol groups of the enzymes GrsA and GrsB. Therefore, purification of the *grsT* gene product and analysis of its enzymatic activity are of interest.

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ADDENDUM IN PROOF

The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. X15577).

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