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# Organization of the Biosynthesis Genes for the Peptide Antibiotic Gramicidin S†

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**A recombinant bacteriophage containing the intact *Bacillus brevis* gene for gramicidin S synthetase 1, *grsA*, and the 5′ end of the gramicidin S synthetase 2 gene, *grsB*, was identified by screening an EMBL3 library with anti-GrsA antibodies. This clone, EMBL315, has a 14-kilobase (kb) insert that hybridizes to the previously isolated 3.9-kb fragment of the *grsB* gene, which encodes the 155-kilodalton ornithine-activating domain of gramicidin S synthetase 2. Deletion and subcloning experiments with the 14-kb insert located the *grsA* structural gene and its putative promoter on a 4.5-kb *Pvu*II fragment which encoded the full-length 120-kilodalton protein in *Escherichia coli*. In addition, hybridization analysis revealed that the 5′ end of the *grsB* gene is located approximately 3 kb from the *grsA* structural gene. Furthermore, these studies indicated that *grsA* and *grsB* are transcribed in opposite orientations.**

The recent cloning of antibiotic biosynthesis genes from the gram-positive bacteria of the genera *Streptomyces* (4, 6, 7, 16, 17) and *Bacillus* (13, 20) and also from the filamentous fungi (3, 24, 25) facilitated studies on the structure, organization, and regulation of these genes at the molecular level (10, 21, 26). Gramicidin S is a cyclic decapeptide antibiotic produced by *Bacillus brevis* in the transition phase from vegetative to stationary growth (19). Gramicidin S biosynthesis is one of the best understood biochemical pathways for a secondary metabolite in *Bacillus* spp. (11, 12) Two multifunctional enzymes, gramicidin S synthetases 1 and 2, with molecular masses of 120 and 280 kilodaltons (kDa), respectively, are involved in the activation, racemization, and condensation of the constituent amino acids via a thiotemplate mechanism (15) to produce the intact peptide antibiotic (Fig. 1). To understand the organization and regulation of the genes involved in the production of peptide antibiotics in *Bacillus* spp., we recently cloned the entire tyrocidine synthetase 1 gene, *tycA*, and studied its regulation in the tyrocidine producer *B. brevis* ATCC 8185 and in the heterologous host *Bacillus subtilis* (20, 21). We also cloned a 3.9-kilobase (kb) DNA fragment of the gramicidin S synthetase 2 gene, *grsB*, which directed the synthesis of a 155-kDa ornithine-activating domain (13) in *Escherichia coli*.

In this work we report on the identification and characterization of a DNA fragment from an EMBL3 library containing the intact gramicidin S synthetase 1 gene, *grsA*, and the 5′ end of the *grsB* gene. We also describe experiments which reveal the location and the direction of transcription for both genes.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and preparation of protein extracts.** *E. coli* JM105 and JM83 (30) were used as hosts for the recombinant plasmids and were grown in L broth medium supplemented with the appropriate antibiotics. For infection with the EMBL3 recombinant bacteriophages, the P2 lysogen *E. coli* P2392, a derivative of LE392 (18), was used (Stratagene, San Diego, Calif.). *E. coli* BHB2688 and BHB2690 were used for the preparation of in vitro packaging extracts and were grown in NZ medium (9, 18). Gramicidin S producer *B. brevis* ATCC 9999 was grown in nutrient broth as described previously (19).

Protein crude extracts were prepared from EMBL315-infected *E. coli* C600 cells (16) grown at 37°C in NZ medium. Cells were collected before complete lysis, usually at an optical density at 600 nm of about 0.8. Preparation of protein extracts and fractionation with 50% ammonium sulfate were done as described by Krause et al. (13). Further purification of the encoded gene product was carried out by anion-exchange fast-performance liquid chromatography on a MonoQ HR5/5 (Pharmacia, Inc.) column with buffer A, containing 20 mM MOPS (3-*N*-morpholinopropanesulfonic acid) (pH 7.2), 500 mM EDTA, and 1 mM dithioerythritol. Proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in buffer A and analyzed by the amino acid-dependent ATP-$PP_i$ exchange reaction (13) and Western blot (immunoblot) analysis (29). For both reactions, 50 μl of each fraction was used.

**Gene bank construction and screening.** Partially *Sau*3A-digested chromosomal DNA from *B. brevis* was fractionated on a 10 to 40% linear sucrose gradient (SW27 rotor [Beckman Instruments, Inc.], at 24,000 rpm for 24 h), and fragments in the range of 10 to 25 kb were ligated into the *Bam*HI sites of EMBL3 vector arms (Stratagene). The ligation mixture was packaged in vitro and used to infect *E. coli* P2392. Packaging extracts were prepared by standard methods as described previously (9). The plaques were screened for the production of proteins immunoreactive with anti-GrsA antibodies immediately after the in vitro packaging step without amplification. Triton X-100-free nitrocellulose filters (HATF; Millipore Corp.) were soaked in an overnight culture of *E. coli* P2392, air dried, and laid onto agar plates containing the recombinant phages. After 15 min of incubation at 4°C, the prints were removed from the master plates and put onto fresh plates. The plaques became visible after 3 to 4 h of incubation at 37°C. The filters were screened in a reaction sequence with anti-GrsA antibodies and $^{125}$I-labeled protein A (30 Ci/mmol; Amersham Corp.) by the method of Helfman et al. (8) with the modifications described by Krause et al. (13).

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† This paper is dedicated to Friedrich Cramer on his 65th birthday.

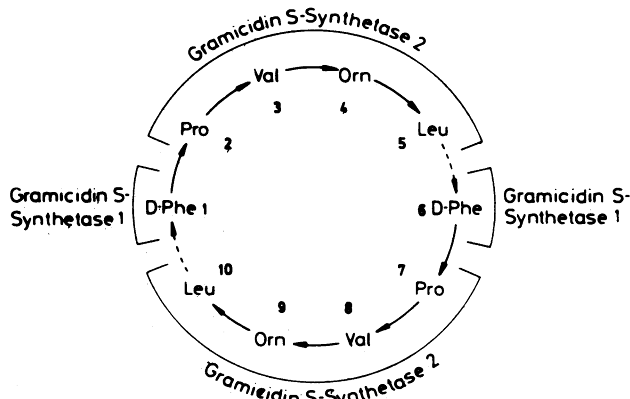


FIG. 1. Amino acid sequence of gramicidin S. The brackets embrance the amino acids activated by the multifunctional enzymes gramicidin S synthetases 1 and 2, which are encoded by the *grsA* and *grsB* genes, respectively.

Positive recombinant phages were purified by reinfection. To identify proteins that were encoded by phage-infected cells or synthesized in *E. coli* cells bearing recombinant plasmids, we prepared as follows total cellular proteins. Cells were converted to spheroplasts by lysozyme treatment in a buffer containing 0.5 mg of lysozyme (Boehringer GmbH) per ml, 50 mM Tris hydrochloride (pH 7.5), and 1 mM dithioerythritol for 15 min at 0°C, disrupted by boiling for 10 min in sodium dodecyl sulfate (SDS) sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (14) and then Western blotting as described by Towbin et al. (29). The detection of immunoreactive proteins was performed by treatment of the blots with anti-GrsA antibodies, incubation with $^{125}$I-labeled protein A, and autoradiography. Alternatively, anti-GrsA antibodies adhering to the filter were detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G, using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as the substrates (2).

**Plasmids, DNA isolation, and in vitro manipulations.** Rapid methods for purifying phages and DNA from the recombinant phages were as described by Silhavy et al. (27). The 14-kb fragment recovered from the recombinant phages was subcloned into pEMBL18 (5), creating the recombinant plasmid pGS1-2. All other recombinant plasmids were derived from pGS1-2 and subcloned into pUC18 or pUC19 (30). Plasmid pMK21 contains the intact *grsA* gene within the 4.5-kb *Pvu*II fragment, whereas plasmids pKE1, pKC2, and pMK18 carry successive deletions at the 3′ end of the *grsA* gene (Fig. 2). pMK9 has a deletion at the 5′ end of *grsA* which abolishes the expression of this gene in *E. coli*. Plasmid pMK2 is an independent isolate which was previously shown to contain a 3.9-kb DNA fragment of the *grsB* gene (13). Chromosomal DNA from *B. brevis* was isolated as described previously (13), and plasmids were prepared from *E. coli* cells by the method of Birnboim and Doly (1). Restriction endonuclease digestions, electroelution of DNA fragments, ligation, nick translation, and other manipulations of DNA were as described by Maniatis et al. (18). Blot hybridization analysis was performed by the method of Southern (28).

## RESULTS

**Cloning and characterization of recombinant phage EMBL315.** Plaque blots of 4,000 recombinant phages containing *B. brevis* genomic DNA in the range of 10 to 25 kb were tested by an in situ immunoassay for the production of gramicidin S synthetase 1 with anti-GrsA antibodies. Three positive recombinant phage clones were purified by two

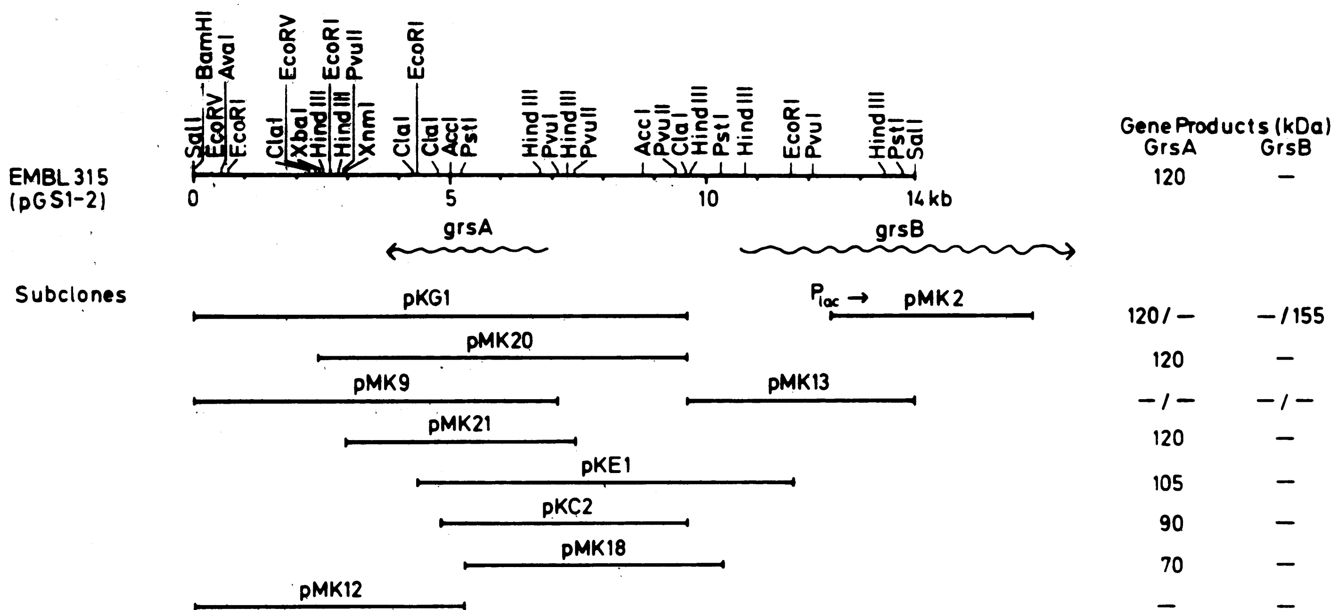


FIG. 2. Restriction endonuclease map of the gramicidin S gene cluster and location and orientation (wavy lines) of the *grsA* and *grsB* genes. The sizes of the immunoreactive gene products encoded by the indicated subclones were determined from the Western blot analysis shown in Fig. 3. Numbers at the right indicate proteins encoded by the corresponding plasmid subclones shown at the left. The cloned 14-kb *B. brevis* genomic DNA in recombinant phage EMBL315 was integrated into pEMBL18 to generate pGS1-2. Plasmid subclones containing segments of the pGS1-2 insert DNA integrated into pUC18 and pUC19 are indicated below the restriction map. Plasmid pMK2 was independently isolated and shown to encode the 155-kDa GrsB fragment from the *lac* promoter (14).

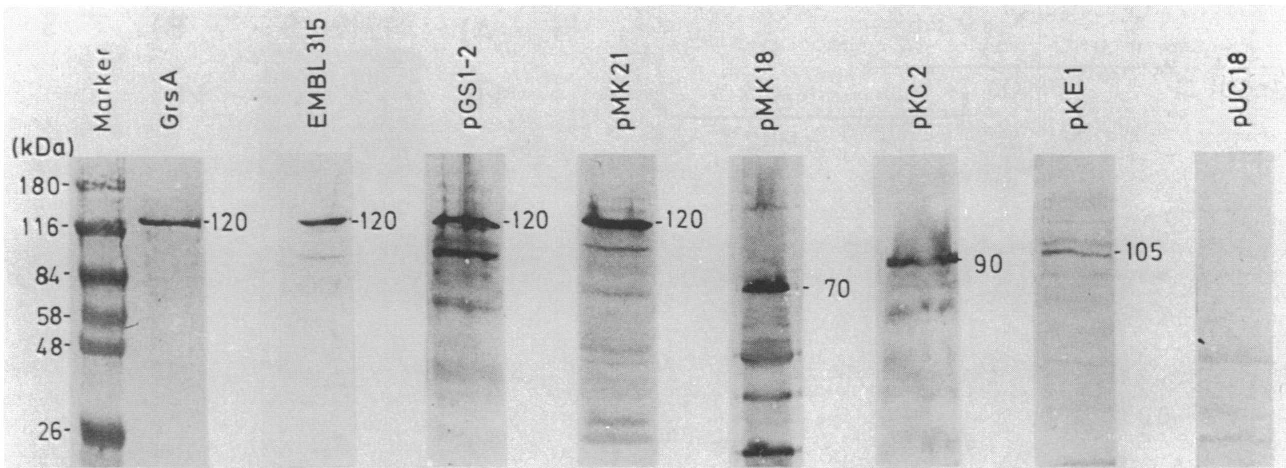


FIG. 3. Western blot analysis of total cellular proteins from *E. coli* cells infected by recombinant lambda phage EMBL315 and cells bearing the indicated plasmid subclones (Fig. 2). Synthesized antigenic proteins were detected with anti-rabbit immunoglobulin and the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate. The GrsA protein from *B. brevis* and a prestained molecular mass protein standard were used as markers.

rounds of infection of P2392 cells and screened again for their ability to produce GrsA antigen. The purified positive phages were tested by Southern hybridization at a high stringency for the presence of DNA sequences homologous to the independently isolated 3.9-kb DNA fragment of the *grsB* gene, which encodes in plasmid pMK2 the ornithine-activating domain of gramicidin S synthetase 2 (13). One recombinant phage, EMBL315, fulfilled both criteria: it produced the GrsA protein as detected by in situ immunoassay, and its 14-kb insert DNA hybridized to the *grsB* gene fragment. These findings suggest that recombinant phage EMBL315 may contain both genes for gramicidin S biosynthesis. Total cellular proteins of EMBL315-infected cells were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot immunodetection analysis. A 120-kDa polypeptide which may represent the *grsA* gene product was identified (Fig. 3). Independent, immunological proof that the 120-kDa protein encoded by *grsA* is indeed gramicidin S synthetase 1 came from enzymatic studies. Total cellular proteins were prepared from EMBL315-infected cells as described in Material and Methods. Protein fractions obtained from the MonoQ column were analyzed for D-phenylalanine-dependent ATP-$PP_i$ exchange, a reaction specific for gramicidin S synthetase 1 (Fig. 4A). The fractions catalizing the highest D-phenylalanine-dependent exchange were also found to contain the 120-kDa GrsA protein, as indicated by Western blot analysis with anti-GrsA antibodies (Fig. 4B).

**Localization of the *grsA* gene and the 5′ end of the *grsB* gene within the cloned DNA.** The 14-kb fragment of *B. brevis* DNA inserted into recombinant phage EMBL315 was recovered by *Sal*I digestion and subcloned into vector pEMBL18 (5), creating the 18-kb recombinant plasmid pGS1-2 (Fig. 2). Both *Sal*I sites flanking the inserted DNA originated from bacteriophage EMBL3. To localize the coding sequence of the *grsA* gene, we constructed a detailed restriction map of the 14-kb DNA fragment and created plasmid subclones containing DNA inserts in the range of 4.5 to 14 kb (Fig. 2). These recombinant plasmids were transformed into *E. coli* JM105 or JM83, and total cellular proteins from mid-log-phase growing cells were subjected to SDS-polyacrylamide gel electrophoresis. Immunoreactive proteins were detected by Western blot analysis with anti-GrsA antibodies. Strains bearing pGS1-2, pMK20, pKG1, or pMK21 directed the synthesis of an immunoreactive protein (independently of the *lac*-promoter located in the vector) which had the same molecular mass (120 kDa) and the same antigenic activity as the *B. brevis* GrsA protein (Fig. 3). These data indicate that

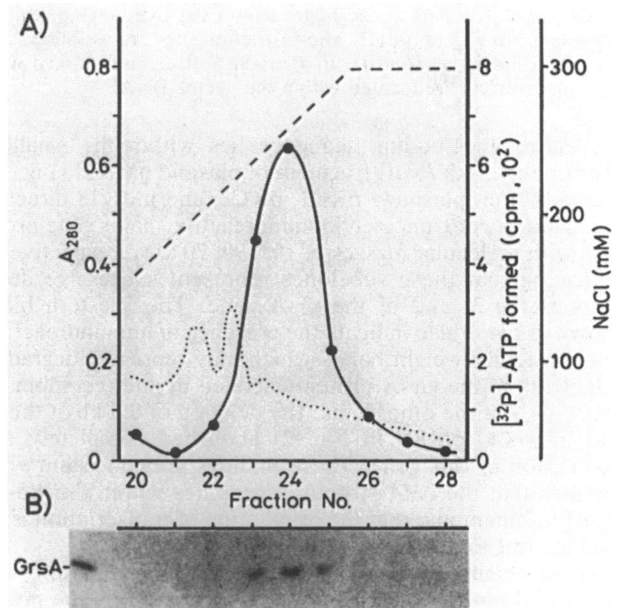


FIG. 4. Protein fractionation of EMBL315-infected *E. coli* cells by fast-performance liquid chromatography on an anion-exchange MonoQ column. Fractions containing GrsA were detected by the D-phenylalanine-dependent ATP-$PP_i$ exchange reaction (A, ●) and immunodetection (Western blot) analysis (B). Proteins of the fractions showing D-phenylalanine-dependent activation were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and subjected to Western blot analysis. The leftmost lane in panel B shows the band of GrsA protein (recognized by anti-GrsA antibodies) that was synthesized in EMBL315-infected cells before fractionation on the MonoQ column. The dotted line represents the $A_{280}$, and the dashed line represents a linear gradient of NaCl.

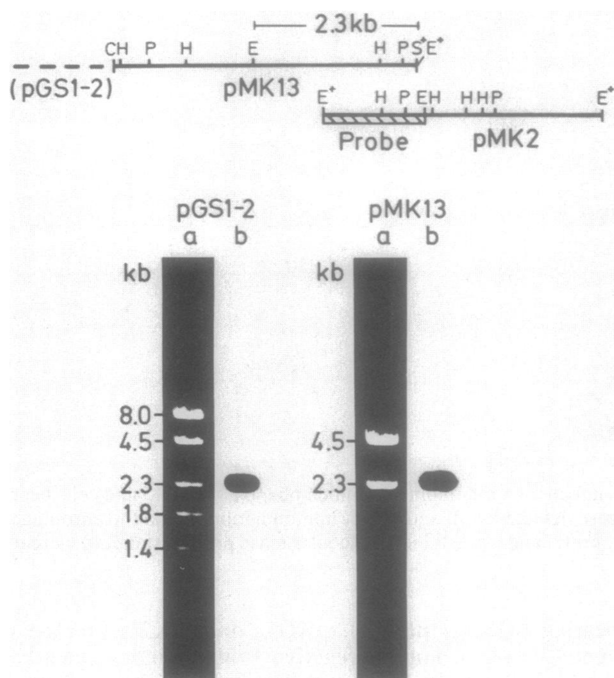


FIG. 5. Southern blot analysis for mapping of the 5′ end of the *grsB* gene within the insert DNA of pGS1-2. DNAs from pGS1-2 and the subclone pMK13 were cleaved with *Eco*RI, resolved on 0.8% agarose gels (lanes a), and transferred to nitrocellulose. The blot was probed with a $^{32}$P-labeled 1.4-kb internal *Eco*RI fragment (hatched bar) of pMK2, and the corresponding autoradiograms are shown in panel b. The top part of the figure shows the overlapping regions between pMK13 and pMK2. The restriction sites are as follows: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. Those marked with plus superscripts are located within the vector DNA.

the entire *grsA*-coding sequence lies within the smallest insert, the 4.5-kb *Pvu*II fragment of plasmid pMK21 (Fig. 2).

In addition, plasmids pKE1, pKC2, and pMK18 directed the synthesis of truncated immunoreactive major gene products with molecular masses of 105, 90, 70 kDa, respectively, indicating that these subclones represent successive deletions at the 3′ end of the *grsA* gene. The Western blots shown in Fig. 3 also indicate the presence of immunoreactive low-molecular-weight bands which may represent degraded fragments of the GrsA protein encoded by the recombinant plasmids. On the other hand, the deletion of 0.5 kb at the 5′ end of *grsA* (pMK9 in Fig. 2) abolished completely the expression of this gene. These findings are consistent with the idea that the *grsA* structural gene lies within the 4.5-kb *Pvu*II fragment and that the orientation of transcription is as indicated in Fig. 2.

To determine the exact location of the *grsB*-coding sequence within the 14-kb DNA fragment, we used the previously isolated *grsB* gene fragment (which encodes only a truncated 155-kDa GrsB protein [the complete GrsB protein is 280 kDa] that activates the nonproteinogenic amino acid ornithine) as a probe in hybridization experiments. *Eco*RI-digested pGS1-2 (Fig. 5) was probed with a $^{32}$P-labeled 1.4-kb internal fragment of the 3.9-kb *grsB* gene fragment. The probe hybridized under stringent conditions only to the 2.3-kb fragment of pGS1-2, which was mapped to the far right end of the 14-kb DNA fragment (Fig. 2 and 4). This location was confirmed by a second hybridization of the *grsB* probe to *Eco*RI-digested pMK13 (Fig. 5). Additional restriction analysis of the inserted DNAs in pMK13 and pMK2

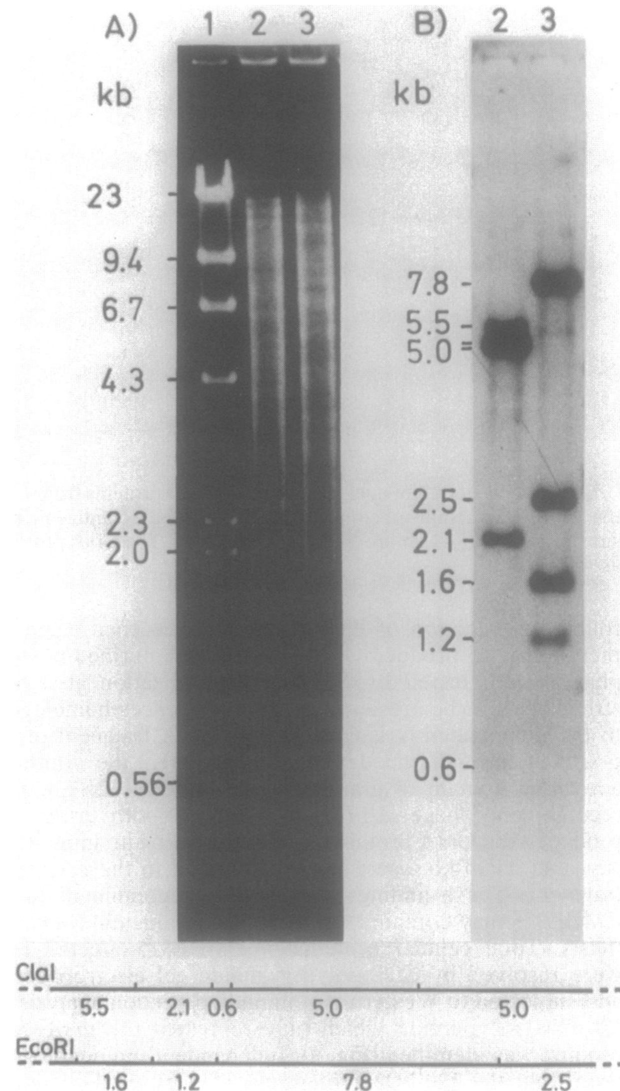


FIG. 6. Southern blot analysis of the chromosomal DNA containing the adjacent *grsA* and *grsB* genes. Chromosomal DNA was cleaved with *Cla*I (lanes 2) and *Eco*RI (lanes 3), resolved on an 0.8% agarose gel (A), and transferred to nitrocellulose. The blot was probed with $^{32}$P-labeled pGS1-2, and the corresponding autoradiogram is shown in panel B. The bottom part of the figure shows the deduced *Cla*I and *Eco*RI restriction maps of the chromosomal *grs* gene cluster and the corresponding clones pGS1-2 and pMK2 (Fig. 2) (14). Lane 1 shows phage lambda *Hin*dIII-digested DNA as size standards.

indicated the presence of a common overlapping sequence of 1.3 kb which contains the *Hin*dIII and *Pst*I sites, which map to the far right end of the 14-kb DNA fragment (Fig. 2 and 5).

Knowing the orientation of the *grsB*-coding sequence within the 3.9-kb fragment in plasmid pMK2 (13) and the region of overlap with the 14-kb fragment, we deduced the orientation of transcription for the *grsB* gene. For complete assurance of the adjacent location of *grsA* and *grsB* genes within the *B. brevis* chromosome, hybridization experiments with genomic DNA were carried out. *Cla*I and *Eco*RI chromosomal digests were probed with $^{32}$P-labeled pGSI-2 (Fig. 6). The probe hybridized under stringent conditions to

five bands in *Cla*I digests and five bands in *Eco*RI digests (Fig. 6B). Also, as expected, the three *Cla*I internal fragments of 5, 2.1, and 0.6 kb and the three *Eco*RI internal fragments of 7.8, 1.6, and 1.2 kb (which were mapped within the 14-kb insert) were all recognized by the probe (Fig. 6). These results are in total agreement with the restriction map of the 14-kb DNA insert and exclude the possibility of DNA rearrangement. The above-mentioned hybridization data and the results of the deletion experiments establish that the gramicidin S biosynthesis genes *grsA* and *grsB* are clustered and transcribed in opposite orientations.

## DISCUSSION

The intact *grsA* gene and the 5′ end of the *grsB* gene, which are involved in the biosynthesis of the peptide antibiotic gramicidin S, were located within a 14-kb DNA fragment of *B. brevis* genomic DNA inserted into recombinant phage EMBL315 and were shown to be organized into two transcriptional units. The exact location of *grsA*, which encodes gramicidin S synthetase 1, was determined by deletion analysis. Plasmid subclones were constructed and tested for the expression of the 120-kDa GrsA protein by Western blot analysis with anti-GrsA antibodies. The 4.5-kb *Pvu*II fragment cloned in pMK21 was identified as the smallest fragment which expressed the full-length 120-kDa protein independently from the *lac* promoter located within the vector. The expression of truncated immunoreactive gene products with molecular masses of 105, 90, and 70 kDa from plasmid subclones pKE1, pKC2, and pMK18 indicated clearly the direction of transcription and located the 3′ end of the *grsA* gene to the left of the *Eco*RI site marking the left end of the inserted DNA in pKE1. The 5′ end of *grsA* may be located within the 0.5-kb *Pvu*II-*Pvu*I fragment, as deletion of this fragment abolished completely the expression of *grsA*. However, the promoter driving *grsA* transcription in *E. coli* may be fortuitous one.

The 5′ end of the *grsB* gene (encoding 280-kDa gramicidin S synthetase 2) was located to the far right end of the 14-kb insert by hybridization experiments. A 1.4-kb internal fragment of the previously isolated 3.9-kb gene fragment of *grsB* (13) hybridized specifically to the far-right 2.3-kb *Eco*RI fragment of the cloned DNA in pGS1-2 and pMK13. This result, in addition to restriction analysis results, indicated the presence of a 1.3-kb DNA in common between the previously isolated 3.9-kb *grsB* gene fragment and the 14-kb insert. The indicated direction of *grsB* transcription, which was divergent from that of *grsA*, was supported by the observation that the expression of the 3.9-kb DNA fragment, which results in the production of the truncated 155-kDa GrsB protein, was driven by the *lac* promoter of the vector (13). Also, the adjacent location of both genes in the *B. brevis* chromosome was verified by hybridizing the 14-kb insert to chromosomal DNA.

These results represent the first evidence at the molecular level for clustering of antibiotic biosynthesis genes in *Bacillus* spp. That this clustering of gramicidin S biosynthesis genes is not the only case in *Bacillus* spp. has been revealed in studies (G. Mittenhuber and M. A. Marahiel, manuscript in preparation) of the organization of the tyrocidine biosynthesis genes *tycA* and *tycB*. In *Streptomyces* spp., molecular cloning experiments have shown that the structural genes for the biosynthesis of the antibiotics methylenomycin (4), actinorhodin (17), streptomycin (23), bialaphos (22), and tylosin (7) are also clustered. Isolation and structural analysis of the biosynthetic genes for the peptide antibiotics produced by *Bacillus* spp. are prerequisites for understanding the regulatory mechanisms that govern the postexponential expression of these secondary metabolites. The characterization of the promoters for *grsA* and *grsB* at the molecular level, which we are currently investigating, may provide further insight into the regulation of gramicidin S production and aid in the elucidation of a possible role for certain stage 0 sporulation regulatory genes in the differential expression of secondary metabolites at the onset of sporulation (21).

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