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Gene Cluster Containing the Genes for Tyrocidine Synthetases 1 and 2 from *Bacillus brevis*: Evidence for an Operon

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From a genomic library of the tyrocidine producer *Bacillus brevis* ATCC 8185 constructed in the bacteriophage vector EMBL3, a recombinant phage which contains the structural genes coding for tyrocidine synthetases 1 and 2, TycA and TycB, was identified. The location of the *tycA* gene within the 16-kilobase insert of this clone, EMBL25-1, was mapped by hybridization studies by using the previously isolated *tycA* DNA as a probe. Restriction analyses, the construction of subclones, and the analysis of proteins encoded by the subclones located the *tycB* gene at the 3′ end of the *tycA* gene and revealed that the two genes are transcribed in the same direction. Nuclease S1 protection studies and DNA sequencing studies of the intergenic region indicated that *tycA* and *tycB* are separated by a 94-base-pair noncoding region and suggested that these genes are organized as an operon.

At the beginning of the stationary growth phase, *Bacillus brevis* ATCC 8185 produces two peptide antibiotics, tyrocidine and linear gramicidin. These compounds are synthesized by a nonribosomal mechanism via the thio-template pathway (18, 20) by the action of multifunctional enzymes. The tyrocidine synthetase system has been studied in detail (22), whereas the enzymes involved in the biosynthesis of linear gramicidin have been only partially purified and characterized (1, 15).

At least three multifunctional enzymes catalyze the synthesis of the cyclic decapeptide tyrocidine (Fig. 1). The substrate amino acid, which is a constituent of the antibiotic, is activated by the corresponding enzyme by the formation of an aminoacyladenylate. These soluble intermediates are transferred to specific thiol groups on and by the multienzyme (20, 24). Mediated by a covalently bound 4′-phosphopantetheine-containing arm (14, 22), peptide synthesis occurs via transthiolation and transpeptidation reactions. In contrast to tyrocidine synthetase 1 (TycA), which initiates tyrocidine biosynthesis, tyrocidine synthetases 2 and 3 (TycB and TycC), which incorporate the subsequent three and six amino acids, respectively, into the antibiotic contain 4′-phosphopantetheine as an integral part.

Genes involved in the biosynthesis of several antibiotics from the genera *Streptomyces* (5, 9, 10) and *Bacillus* (17, 28) and from filamentous fungi (4, 33) have been cloned, and some have been sequenced. The genes involved in polyketide (25, 26) and peptide (16) antibiotic production were clustered in the genome of the producer.

The structural gene encoding TycA was previously isolated on the recombinant plasmid pBT2 (28); *tycA* and the 5′-untranslated region were sequenced (37), and the regulation of the *tycA* expression in the heterologous host *Bacillus subtilis* was studied (29). *tycA* was shown to be expressed from a σ^{43}-like promoter. Its transcription is dependent on the Spo0A, Spo0B, and Spo0E gene products, whereas a mutation in the suppressor locus *abrB* relieved the inhibition of *tycA* transcription in *spo0A* and *spo0B* backgrounds. This paper describes the isolation and characterization of a recombinant bacteriophage which harbors the structural genes for TycA and TycB within a 16-kilobase (kb) DNA fragment. Also, results of experiments which reveal that the two genes are adjacent and which suggest that they are part of an operon are presented.

MATERIALS AND METHODS

Bacterial strains and bacteriophage, growth conditions, and nucleic acid extractions. The tyrocidine producer *B. brevis* ATCC 8185 was grown in nutrient broth, harvested at the end of the logarithmic growth phase, and used for the preparation of chromosomal DNA as described previously (17, 28). For the extraction of RNA, *B. brevis* was grown in Hanson's sporulation medium (30). RNA was prepared from 50-ml culture aliquots as described by Marahiel et al. (29).

Escherichia coli JM105 was routinely used as a host for recombinant plasmids and was grown in 200 ml of 2×YT broth supplemented with ampicillin (50 μg/ml) for the isolation of plasmid DNA by the alkaline lysis method (2). The plasmids were purified by banding in CsCl gradients prior to DNA sequencing (27).

For the preparation of in vitro packaging extracts, *E. coli* BHB2688 and BHB2690 were grown in NZ medium (13). *E. coli* P2392 (Stratagene, San Diego, Calif.), a P2 prophage-containing derivative of strain LE392, was used in the screening experiments and for the propagation of recombinant phage. The bacteriophage were removed from the culture supernatant by polyethylene glycol precipitation and centrifugation in glycerol step gradients (34). DNA was extracted from the phage pellet and purified as described by Silhavy et al. (34).

Gene bank construction, antibodies, and in situ immunoassay. Chromosomal DNA from *B. brevis* ATCC 8185 was partially digested with *Sau*3A. DNA fragments (10 to 22 kb) were ligated into the *Bam*HI site of the bacteriophage vector EMBL3, followed by an in vitro packaging reaction, as described previously (13, 16).

Polyclonal antibodies directed against gramicidin S synthetase 2 (GrsB) were provided by H. J. Skarpeid (University of Oslo, Norway). Anti-gramicidin S synthetase 1 (GrsA) antibodies were supplied by M. Stöffler-Meilicke (Max-Planck-Institut für Molekulare Genetik, Berlin, Federal Republic of Germany). Both antisera were raised in

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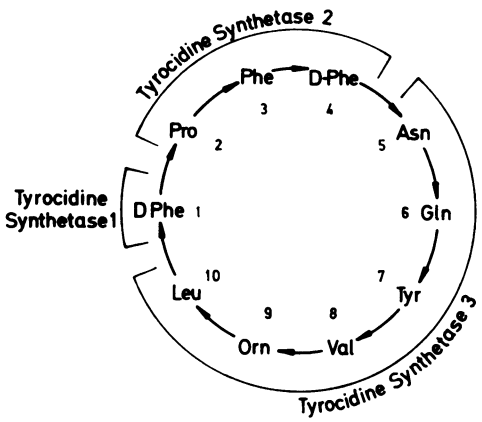

FIG. 1. Amino acid sequence of tyrocidine. The brackets indicate the amino acids activated by the multifunctional enzymes tyrocidine synthetases 1, 2, and 3.

rabbits. The conditions for the in situ immunoassay using anti-GrsB antibodies, followed by incubation with ^{125}I-protein A, were as described previously (12, 17).

Analytical procedures. For the analysis of proteins produced by phage-infected cells or *E. coli* harboring recombinant plasmids, the cell pellets were lysed by treatment with lysozyme and boiled in the presence of sodium dodecyl sulfate (SDS) as described by Krause and Marahiel (16). These crude extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels (19). Proteins were either stained with Coomassie blue R or transferred to nitrocellulose sheets (36). Immunoreactive proteins were detected by incubating the filter with the primary antibody, followed by an incubation step with ^{125}I-protein A and subsequent autoradiography (17). Alternatively, the filter was treated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and developed by using 5-bromo-4-chloro-indolylphosphate as the substrate and Nitro Blue Tetrazolium as an enhancer of the color reaction (3). For the construction of subclones, restricted DNA fragments were separated by electrophoresis in 0.8% low-melting-point agarose gels (7). The desired fragments were cut out and cloned into pUC18 (39) or pGEMZ (Promega Biotec, Madison, Wis.) restricted with the appropriate enzyme.

Conditions for restriction endonuclease digestions, ligations, nick translations, Northern (RNA) and Southern hybridization, electroelution of restriction fragments, 5′-end labeling of the DNA fragments, DNA-RNA hybridization in formamide buffer, and nuclease S1 digestion of the resulting DNA-RNA hybrids were as described by Maniatis et al. (27).

Nonradioactive labeling of DNA fragments with digoxigenin desoxyuridine and detection of DNA-DNA hybrids was performed by using a DNA labeling and detection kit from Boehringer Mannheim. PAGE of the nuclease S1 digestion reactions in 4.0% polyacrylamide gels containing 6 M urea was performed as described by Marahiel et al. (29). Plasmid DNA was sequenced by the method of Chen and Seeburg (6) by using a Sequenase kit (version 2.0) from U.S. Biochemical Corp.

Plasmids. Recombinant plasmids used in this study are listed in Table 1.

RESULTS

Detection and characterization of EMBL25-1. Gramicidin S is a decapeptide antibiotic produced by *B. brevis* ATCC 9999 by a pathway similar to that of tyrocidine biosynthesis (18, 20). Antibodies to gramicidin S synthetase 2 (GrsB) exhibit cross-reactions with all three tyrocidine synthetases (D. Bothe, Ph.D. thesis, Technische Universität Berlin, 1986).

Approximately 4,500 recombinant phage containing inserts of *B. brevis* ATCC 8185 DNA were subjected to an in situ immunoassay using anti-gramicidin S synthetase (GrsB) antibodies. Three positive clones were detected and isolated by two reinfection steps of *E. coli* P2392 and by repetitions of the immunoassay. One clone was characterized further.

Proteins from a liquid culture infected with this clone, EMBL25-1, were subjected to SDS-PAGE on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose. Immunoreactive proteins recognized by anti-GrsB antibodies were observed in the cell lysate (G. Mittenhuber, M. Krause, and M. A. Marahiel, *in* L. O. Butler, C. Harwood, and B. E. B. Moseley (ed.), *Genetic Transformation and Expression*, in press). The filters were incubated with either anti-GrsB or anti-gramicidin S synthetase 1 (GrsA) antibodies, treated with ^{125}I-labeled protein A, and autoradiographed. Anti-GrsA antibodies recognize only the homologous enzyme of the tyrocidine synthetase complex, tyrocidine synthetase 1 (TycA; 122.6 kilodaltons [kDa]), and show no cross-reactions with the other two proteins (Bothe, Ph.D. thesis). As shown in Fig. 2, anti-GrsB antibodies exhibited a cross-reaction with two proteins in the range of 120 and 190 kDa, whereas anti-GrsA antibodies detected only the 120-kDa protein. In addition, a band around 90 kDa which cross-reacts with anti-GrsA more than with anti-GrsB antibodies is visible in Fig. 2, lanes 2 and 3. Interestingly, we

TABLE 1. Plasmids used in this study

Plasmid	Insert size (kb)	Gene product	Derivation	Reference(s)
pBT2	5.2	Intact (122.6-kDa) TycA	*B. brevis* chromosomal DNA	28, 37
pGC22	3.3	Intact (122.6-kDa) TycA	pBT2	29
pMS8	4.3	None detected	EMBL25-1	This paper
pMH46	3.0	None detected	EMBL25-1	This paper
pMS1	7.0	TycB fragment (190 kDa), constitutive expression in *E. coli*	EMBL25-1	This paper
pMS9	7.0	TycB fragment (190 kDa), overexpression under control of the *lac* promoter	EMBL25-1	This paper
pMS11	3.6	Truncated TycB fragment (130 kDa), expression under control of the *lac* promoter	pMS9	This paper
pMH1	2.5	Truncated TycB fragment (90 kDa), expression under control of the *lac* promoter	pMS9	This paper
pMA1	1.1	None detected, used for DNA sequencing	pMS9	This paper

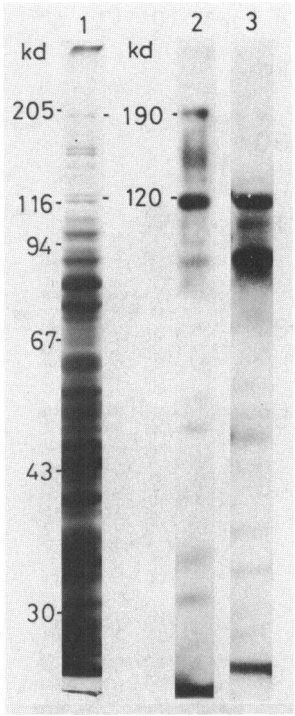

FIG. 2. Western blot analysis of EMBL25-1-infected cells. Proteins were separated by SDS-PAGE on a 7.5% polyacrylamide gel. Lane 1 shows the Coomassie blue R-stained gel. Proteins in lanes 2 and 3 were transferred to nitrocellulose filters and incubated with anti-GrsB (lane 2) or anti-GrsA (lane 3) antibodies. Immunoreactive proteins of 120 and/or 190 kDa were detected by treatment with ^{125}I-labeled protein A (Amersham; 30 mCi/mg) and by autoradiography.

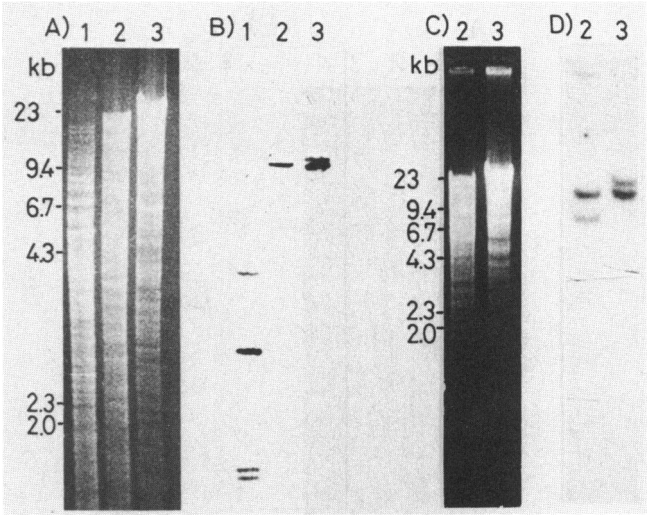

FIG. 3. Southern blot analysis of the chromosomal DNA from *B. brevis* ATCC 8185 containing the *tycA* and *tycB* genes. Chromosomal DNA was completely digested with *Hin*dIII (lane 1), *Sal*I (lane 2), or *Sma*I (lane 3), separated on a 0.8% agarose gel (panels A and C), and transferred to nitrocellulose. Panel B shows Southern blot hybridization of the gel shown in panel A, with digoxigenin desoxyuridine-labeled *Sst*I/*Sal*I fragment DNA from plasmid pMS1 as the probe. The blot corresponding to the gel shown in panel C was allowed to hybridize with ^{32}P-labeled EMBL25-1 DNA. The autoradiogram is shown in panel D.

also detected a band in this molecular mass range and the same antigenic features in protein extracts obtained from another bacteriophage clone (data not shown). In light of this observation, we assume that the 90-kDa band is a degradation product of the 120-kDa protein. To identify the nature of the cross-reacting material, proteins from a large-scale preparation of EMBL25-1-infected cells (400 ml of culture) were fractionated by gel filtration on AcA34 and ion-exchange chromatography on Sephadex DE52 and assayed for enzyme activities which are specific for tyrocidine biosynthesis. These experiments revealed that the 120-kDa protein represents tyrocidine synthetase 1 and that the 190-kDa protein contains the proline- and L-phenylalanine-activating domain of tyrocidine synthetase 2 (H. von Döhren, personal communication).

The 16-kb insert of EMBL25-1 was mapped by using the restriction endonucleases *Hin*dIII, *Sal*I, *Sma*I, and *Sst*I (see Fig. 5). In order to ensure that the 16-kb insert represents a chromosomal DNA fragment of *B. brevis* ATCC 8185, chromosomal DNA from the tyrocidine producer was completely digested with *Sal*I or *Sma*I. Each enzyme has a single recognition site within the insert of EMBL25-1. The reaction products were separated on a 0.8% agarose gel in TAE buffer (27), blotted onto nitrocellulose, and hybridized with ^{32}P-labeled EMBL25-1 DNA. In each lane of the corresponding autoradiogram (Fig. 3D), two separate signals in the high-molecular-mass range were detected. In addition, gel-fractionated chromosomal DNA of *B. brevis* ATCC 8185 digested with *Hin*dIII, *Sal*I, or *Sma*I was hybridized with digoxigenin desoxyuridin-labeled *Sst*I/*Sal*I DNA fragments from plasmid pMS1 (Fig. 3B). The hybridization pattern was as predicted by the restriction map of the EMBL25-1 insert (see Fig. 5). These observations prove that the insert of EMBL25-1 represents a genomic DNA fragment and that it did not emerge from a cloning artifact during the construction of the gene bank.

Mapping of the *tycA* and *tycB* genes and determination of the direction of transcription. In previous studies, the *tycA* gene was mapped and subcloned on a 3.3-kb *Hin*cII fragment within the 5.2-kb insert of pBT2, resulting in plasmid pGC22 (29). EMBL25-1 DNA was restricted with *Hin*cII, resolved in an agarose gel, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled pBT2 DNA. The 3.3-kb *Hin*cII fragment containing the *tycA* gene was detected in the autoradiogram (data not shown).

The *tycA* gene was mapped on the insert of EMBL25-1 in Southern blot experiments by using ^{32}P-labeled pBT2 DNA as a probe (Fig. 4 and 5). The vector EMBL3 has one *Hin*dIII site in the right arm and two *Sal*I sites flanking the cloned insert. As shown in Fig. 4 (lane 3), EMBL25-1 DNA cut with *Hin*dIII alone exhibits three signals in the corresponding autoradiogram: a 1,170-base-pair (bp) fragment, which was mapped within the *tycA* gene (28, 37); a 3.0-kb fragment; and a large fragment around 22 kb, which represents the left arm of the vector and a part of the insert. As expected, radioactively labeled pBT2 DNA detects a fragment (3.2 kb) which defines the distance from the *Sal*I site at the left arm of the vector to the first *Hin*dIII site within the insert of EMBL25-1 in a *Hin*dIII/*Sal*I double digestion of EMBL25-1 DNA (lane 4). Restriction of EMBL25-1 DNA with *Sal*I reveals that the *tycA* gene is located on a 12-kb *Sal*I fragment (lane 2).

These data allowed us to map the *tycA* gene at close proximity to the left arm of the vector within the insert of EMBL25-1. To determine the location of the *tycA* gene within EMBL25-1, comparative restriction analyses of

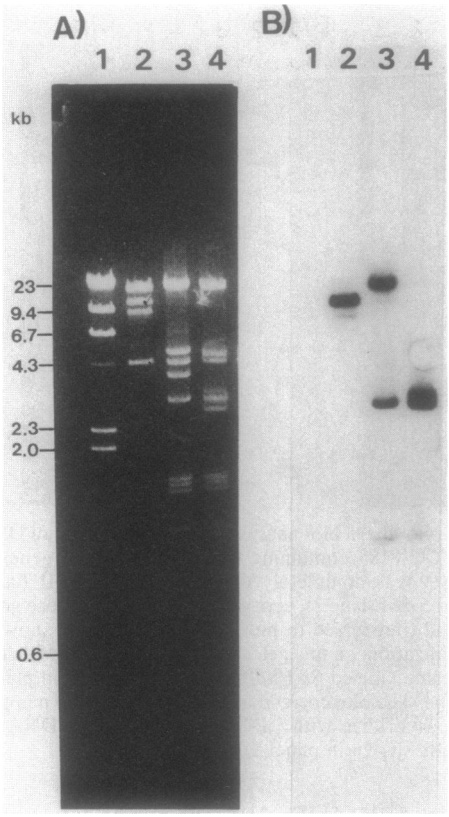

FIG. 4. Southern blot analysis for mapping the *tycA* gene within the insert of EMBL25-1. EMBL25-1 DNA was restricted with *Sal*I (lane 2), *Hin*dIII (lane 3), or *Sal*I and *Hin*dIII (lane 4), subjected to electrophoresis in a 0.8% agarose gel (panel A), and transferred to nitrocellulose. The blot was probed with ^{32}P-labeled pBT2 DNA; the corresponding autoradiogram is shown in panel B.

pGC22 and the EMBL25-1-derived subclone pMH46, which contains the 3′ end of the *tycA*, were performed. The direction of transcription of the *tycA* gene was previously determined (29, 37) and is indicated in Fig. 5.

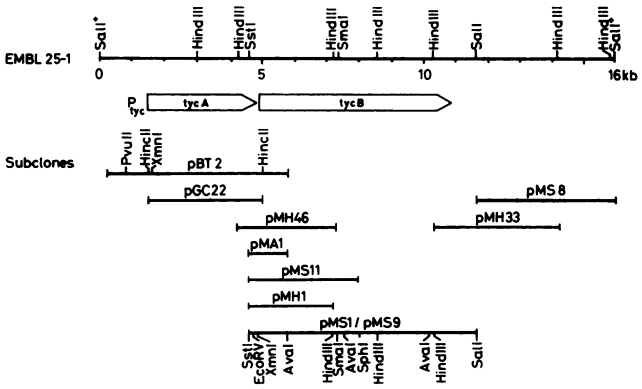

FIG. 5. Restriction map of the insert of EMBL25-1 and of subclones. The relative positions of the independently isolated plasmids pBT2 and pGC22 and the direction of transcription (arrows) of the *tycA* and *tycB* genes are shown. In plasmid pMS9, the *tycB* gene is under control of the *lac* promoter, whereas plasmid pMS1 represents the other orientation. The *Sal*I sites flanking the DNA insert and which are marked by a plus sign (+) originate from the vector EMBL3.

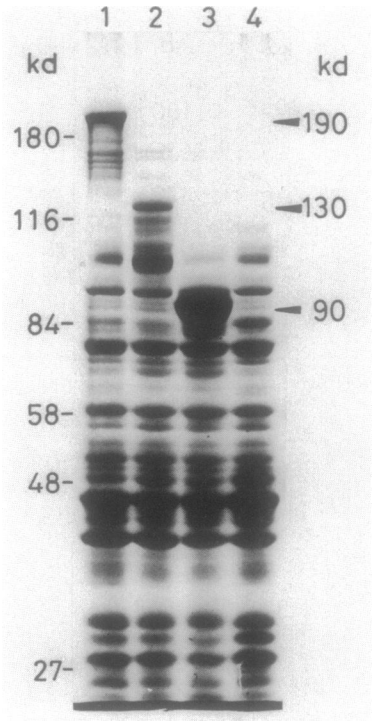

FIG. 6. Analysis of the proteins encoded by the subclones pMS9, pMS11, and pMH1. A 7.5% polyacrylamide gel stained with Coomassie blue R is shown. Lane 1 contains proteins from cells bearing pMS9; lanes 2 and 3 contain proteins from cells harboring pMS11 and pMH1, respectively. The arrowheads at the right margin mark the proteins encoded by the recombinant plasmids. Lane 4 contains proteins from cells harboring pMA1, a plasmid which was used for DNA sequencing of the intergenic region (Table 1). Cells were induced with IPTG. Protein expression from cells harboring the recombinant plasmids was also tested under repressing conditions (addition of 10 mM glucose). No plasmid-encoded proteins were detected.

To determine the location of the *tycB* gene on EMBL25-1, a series of subclones was constructed (Fig. 5). Plasmid pMS1, a derivative of the vector pGEMZ bearing the 7.0-kb *Sst*I/*Sal*I fragment, was found to express the 190-kDa TycB fragment weakly (data not shown). In order to clone the *Sal*I/*Sst*I fragment in the opposite orientation with respect to the inducible *lac* promoter of the vector, the pUC18 derivative of pMS9 was constructed. This plasmid harbors the *lac* promoter adjacent to the *Sst*I site of the 7.0-kb *Sst*I/*Sal*I fragment. Production of the 190-kDa protein in cells bearing pMS9 is inducible by the addition of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture (final concentration, 1 mM) (Fig. 6, lane 1), and it can be repressed by subjecting the cells to catabolite repression, i.e., by the addition of 10 mM glucose. IPTG induction even results in overproduction of the 190-kDa protein. No such effects were observed in cells harboring pMS1 (data not shown).

These results and those previously reported (29, 37) indicate that the *tycA* and *tycB* genes are transcribed in the same direction. The direction of transcription of the *tycB* gene was confirmed by the analysis of proteins expressed by the plasmids pMS11 and pMH1. These derivatives of pMS9 carry deletions at the putative 3′ end of the *tycB* gene. Both pMS11 and pMH1 caused production of truncated TycB protein (130 and 90 kDa, respectively) after IPTG induction (Fig. 6, lanes 2 and 3). The 130-kDa protein was recognized

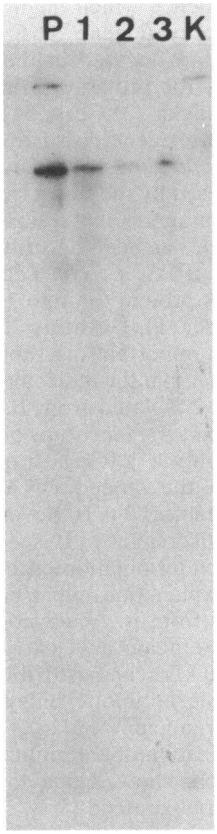

FIG. 7. S1 nuclease analysis of the intergenic region. The 1-kb *Sst*I/*Ava*I fragment which contains the 3′ end of *tycA* and the 5′ end of *tycB* was labeled at the 5′ end and hybridized with total RNA from *B. brevis* isolated at different growth stages. The digestion products were separated on a 4.0% acrylamide–urea gel. Lane P contains a portion of the labeled DNA probe, lanes 1, 2, and 3 contain the S1 digests of the hybridization reactions (lane 1, RNA prepared at t_0; lane 2, RNA extracted at t_1; lane 3, RNA prepared at t_3), and lane K contains the control, in which yeast tRNA was hybridized with the probe and digested with nuclease S1.

by the anti-GrsB antibodies, whereas the 90-kDa fragment did not cross-react with these antibodies (data not shown).

The *Sal*I/*Sst*I fragment was mapped for the restriction enzymes *Acc*I, *Ava*I, *Eco*RV, *Hin*dIII, *Sph*I, and *Xmn*I (Fig. 5).

Characterization of the intergenic region. DNA sequencing studies revealed that the *Sst*I site of plasmid pBT2, which corresponds to the *Sst*I site of plasmids pMS1 and pMS9, is located at the 3′ end within the coding region of the *tycA* gene (37) (see Fig. 8).

An *Ava*I site was mapped downstream of the *Sst*I site within the *tycB* gene (Fig. 5). To determine whether a transcriptional start point is present at the 5′ end of the *tycB* gene, this 1-kb *Sst*I/*Ava*I fragment was isolated and labeled at its 5′ end by using T4 polynucleotide kinase and [γ-^{32}P]ATP.

The labeled fragment was hybridized with total *B. brevis* RNA prepared at different growth stages (t_0, t_1, and t_3), and the hybridization products were subjected to nuclease S1 digestion. Full protection of the labeled fragment was observed after PAGE, whereas the control (hybridization of the fragment with yeast tRNA) was completely digested by nuclease S1 (Fig. 7).

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                ---tycA►
3381  GAG CTC GTC CTC ACA TTC TCT TAC AGC TCG GAG CAG TAT CGG
      Glu Leu Val Leu Thr Phe Ser Tyr Ser Ser Glu Gln Tyr Arg
       Sst I
3423  GAA GAG TCC ATC CAG CAA TTG AGC CAA AGT TAT CAA AAG CAT
      Glu Glu Ser Ile Gln Gln Leu Ser Gln Ser Tyr Gln Lys His

3465  CTG CTT GCC ATC ATC GCG CAT TGC ACC GAG AAA AAA GAA GTA
      Leu Leu Ala Ile Ile Ala His Cys Thr Glu Lys Lys Glu Val

3507  GAG CGA ACG CCC AGC GAT TTC AGC GTC AAA GGT CTC CAA ATG
      Glu Arg Thr Pro Ser Asp Phe Ser Val Lys Gly Leu Gln MET

3549  GAA GAA ATG GAC GAT ATC TTC GAA TTG CTT GCA AAT ACA CTG
      Glu Glu MET Asp Asp Ile Phe Glu Leu Leu Ala Asn Thr Leu

3591  CGC TAA ACAGATGTTGGCCACCATTTTCAGGGGCAACTGCGTGCTTTCATTCC
      Arg ***
                                                  SD        tycB►
3644  CATTTTTATACATTTATAACAAATAAAGATATATCCGAGGTGCCGTA ATG AGT
                                                        Met Ser

3697  GTA TTT AGC AAA GAA CAA GTT CAG GAT ATG TAT GCG TTG ACC
      Val Phe Ser Lys Glu Gln Val Gln Asp MET Tyr Ala Leu Thr

3739  CCG ATG CAA GAG GGG ATG CTG TTT CAC GCC TTG CTC GAT CAA
      Pro MET Gln Glu Gly MET Leu Phe His Ala Leu Leu Asp Gln

3781  GAG CAC AAC TCG CAT CTG GTA CAG ATG TCG ATT TCG TTG CAG
      Glu His Asn Ser His Leu Val Gln MET Ser Ile Ser Leu Gln

3823  GGC GAT CTT GAC GTT GGG CTA TTT ACG GAT AGC CTG CAT GTG
      Gly Asp Leu Asp Val Gly Leu Phe Thr Asp Ser Leu His Val

3865  CTG GTA GAG AGA TAC GAT GTA TTC CGC ACG TTG TTT CTC TAT
      Leu Val Glu Arg Tyr Asp Val Phe Arg Thr Leu Phe Leu Tyr

3907  GAA AAG CCT GAA GCC AGC CCT TTG CAA GTT GTC TTG AAG CCA
      Glu Lys Pro Glu Ala Ser Pro Leu Gln Val Val Leu Lys Pro

3949  ACG CGC CTA TTC CGA TCG AAT TTT ACG CAC TTG CCC TGC CTG
      Thr Arg Leu Phe Arg Ser Asn Phe Thr His Leu Pro Cys Leu

3991  CGC ACG AGT CCG AGA AAC AAC TTC GCT ATA CGC AAT ACA AAG
      Arg Thr Ser Pro Arg Asn Asn Phe Ala Ile Arg Asn Thr Lys

4033  CGC GAT CAG GAG CGC ACG TTT CAT CTG GCA AAA GAC CCG TTG
      Arg Asp Gln Glu Arg Thr Phe His Leu Ala Lys Asp Pro Leu

4075  CAT GCC GGT GCC TTT TCC AAA TGT CCC AGC GGA CTA CAG GTC
      His Ala Gly Ala Phe Ser Lys Cys Pro Ser Gly Leu Gln Val

4117  ATC TGG AGC TTT CAT CAC ATC CTC ATG GAC GGC TGG TGC TCC
      Ile Trp Ser Phe His His Ile Leu MET Asp Gly Trp Cys Ser

4159  AGC ATT ATT TTT GAG TAC CTG CTT GCC ATC TAC TTG TCC TTG
      Ser Ile Ile Phe Glu Tyr Leu Leu Ala Ile Tyr Leu Ser Leu

4201  CAA AAG AAG ACG GCA CTC TCC CTG GAG CCC GTA CAG CCA TAC
      Gln Lys Lys Thr Ala Leu Ser Leu Glu Pro Val Gln Pro Tyr

4243  AGT CGC TTT ATC AAC TGG CTG GAA AAA CAA AAT AAA CAG GCC
      Ser Arg Phe Ile Asn Trp Leu Glu Lys Gln Asn Lys Gln Ala

4285  GCT CTC AAC TAT TGG AGC GAC TAT CTG GAA GCC TAT GAA CAA
      Ala Leu Asn Tyr Trp Ser Asp Tyr Leu Glu Ala Tyr Glu Gln

4327  AAG ACT ACC TTG CCG AAG AAG GAA GCT GCC TTC GCC AAA GCA
      Lys Thr Thr Leu Pro Lys Lys Glu Ala Ala Phe Ala Lys Ala

4369  TTT CAA CCA ACC CAA TAC CGC TTT TCG CTG AAC CGC ACC TTG
      Phe Gln Pro Thr Gln Tyr Arg Phe Ser Leu Asn Arg Thr Leu

4411  ACC AAG CAG CTC GGG
      Thr Lys Gln Leu Gly
                 Ava I
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FIG. 8. Nucleotide and predicted amino acid sequences of the 1,145-bp *Sst*I/*Ava*I fragment overlapping the 3′ end of *tycA* and the 5′ end of *tycB*. Nucleotide positions are numbered relative to the previously mapped transcriptional start site at the front of the *tycA* gene (29). A putative ribosomal binding site identified 7 bp upstream of the *tycB* open reading frame is underlined. The complete nucleotide sequence of *tycA* (3,261 bp) has been previously determined (37).

This observation suggested that no transcription initiation signals, which are used by *B. brevis* RNA polymerase, are present on this *Sst*I/*Ava*I fragment and that the *tycA* and *tycB* genes might be expressed as an operon, i.e., as polycistronic mRNA from the *tycA* promoter (29). To confirm this hypothesis, the nucleotide sequence of the 1-kb *Sst*I/*Ava*I fragment containing the intergenic region between *tycA* and *tycB* was determined (Fig. 8). The *Sst*I restriction site is

211 bp upstream of the termination codon (TAA) of the *tycA* gene and 305 bp from the presumptive translational initiation codon (ATG) of the *tycB* gene, which is followed by an open reading frame extending to at least 735 bp (the extent of our sequence analysis [Fig. 8]). The *tycA* structural gene and the noncoding intergenic region between the 3′ end of the *tycA* gene and the 5′ end of the *tycB* gene is 94 bp and reveals no consensus sequences known to bind RNA polymerases in *Bacillus* spp. In addition, Northern blot analysis using total RNA from *B. brevis* ATCC 8185 isolated around t_0 and a *tycA* labeled probe revealed the presence of a DNA-RNA hybrid which smears in the range of 10 to 6 kb, which is definitively larger than the 3-kb transcript of *tycA* alone (data not shown). This suggests that *tycA* and *tycB* might be transcribed as one unit. However, an initiation point for the transcription from within the *tycA* structural gene cannot be excluded.

DISCUSSION

From a genomic library of *B. brevis* ATCC 8185 constructed in the bacteriophage vector EMBL3, a recombinant phage containing the structural genes for the tyrocidine synthetases 1 and 2 (TycA and TycB) was isolated and characterized. The presence of the *tycA* gene on EMBL25-1 DNA was confirmed by several independent experiments. In protein extracts prepared from EMBL25-1-infected *E. coli* cells, enzymatic activities specific for tyrocidine synthetase 1, i.e., D-phenylalanine-dependent ATP/PP_i exchange (H. von Döhren, personal communication), were detectable and in Western blots (immunoblots) of these extracts treated with either anti-GrsA or anti-GrsB antibodies an immunoreactive protein with the same molecular mass as the TycA protein was identified.

The entire *tycA* gene was mapped previously on a 3.3-kb *Hin*cII fragment. We demonstrated in Southern blots that the same fragment is present in *Hin*cII-digested EMBL25-1 DNA. The conclusion that EMBL25-1 harbors also the *tycB* structural gene is based on the analysis of protein extracts infected with EMBL25-1. Enzymatic activities specific for tyrocidine synthetase 2 were detected in the chromatography fractions containing the 190-kDa protein (H. von Döhren, personal communication) which exhibits a cross-reaction with anti-GrsB antibodies and not with anti-GrsA antibodies. The specific reaction of anti-GrsA antibodies with TycA can be explained on the basis of the functional homology of both enzymes (both activate and epimerize the amino acid phenylalanine) and by the strong homology of the corresponding genes, as indicated on the DNA sequence level (J. Krätzschmar, M. Krause, and M. A. Marahiel, submitted for publication).

By constructing subclones and by using the independently isolated *tycA* gene as a probe, the *tycB* and *tycA* genes were mapped on EMBL25-1 and the direction of transcription of both genes was determined. Nuclease S1 protection experiments and DNA sequencing of the region between the 3′ end of *tycA* and the 5′ end of *tycB* revealed that the transcription of *tycB* does not initiate within this fragment and suggest that both genes might be transcribed as one unit. Also, the fact that in Northern blot analysis a transcript of at least 6 kb was observed (data not shown) supported this idea. The complete nucleotide sequence for the *tycA* gene and more than 700 bp of the 5′ end of the *tycB* gene have been determined (37) (Fig. 8). The initiation codon of the *tycB* gene is 94 bp downstream of the 3′ end of the *tycA* gene. Upstream of the putative start codon of the *tycB* gene, a ribosome binding site was identified. A sequence which might be responsible for the expression of the *tycB* gene in *E. coli* cells bearing plasmid pMS1 should be located in the intergenic region. Consensus sequences for promoters functional in *E. coli* investigated by Studnicka (35) can be also found in the coding region of the *tycA* gene downstream of the *Sst*I site and in the intergenic region. A fortuitous promoter sequence, which is not used by the transcriptional apparatus of *B. thuringiensis* mediating the expression of *B. thuringiensis* crystal toxin in *E. coli*, has been described (38).

After induction by IPTG, *E. coli* cells harboring pMS9 overproduce the TycB protein to up to 20% of total cellular protein (G. Mittenhuber, Ph.D. thesis, Technische Universität Berlin, 1988). An important discrepancy which has yet to be resolved is the fact that the molecular mass of the intact tyrocidine synthetase 2 isolated from *B. brevis* is 230 kDa and that the recombinant *E. coli* strains or cells infected with EMBL25-1 produce only a 190-kDa fragment of the TycB protein, even though the cloned DNA fragment is long enough to code for the intact TycB. Several factors might be responsible for this discrepancy. It seems possible that a premature transcription termination of the *tycB* gene occurs in *E. coli*. Another explanation might be that the 230-kDa TycB protein isolated from *B. brevis* contains a covalently bound 4′-phosphopantetheine acyl carrier protein (14, 21) and that the missing 40 kDa can be attributed to this protein.

Clustering of peptide antibiotic biosynthesis genes was also described for gramicidin S (16). Numerous studies focusing on the organization of antibiotic biosynthesis genes from *Streptomyces* spp. showed that these genes are also clustered in the bacterial genome (5, 8, 10, 25, 26, 31, 32).

We have constructed plasmids which carry deletions at the 3′ end of the *tycB* structural gene and encode truncated TycB proteins. The 130-kDa TycB fragment encoded by plasmid pMS11 was subjected to proteolysis. Beside the main band around 130 kDa, degradation products around 100 kDa were visible on the Western blot (data not shown). The proteolysis of incomplete polypeptide chains in *E. coli* was studied by using β-galactosidase as a model system (11, 23). The 90-kDa TycB fragment encoded by plasmid pMH1 exhibits a very low antigenicity. This observation might be explained by presuming that most of the polyclonal antibodies recognize the C-terminal region of TycB, a part which is supposed to be at the surface of the protein.

The analysis of these protein fragments at the enzymatic level, as well as the analysis of the other two bacteriophage clones that we isolated, might provide further information on the structure/function relationship of tyrocidine synthetases.

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