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The Tyrocidine Biosynthesis Operon of *Bacillus brevis*: Complete Nucleotide Sequence and Biochemical Characterization of Functional Internal Adenylation Domains

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The cyclic decapeptide antibiotic tyrocidine is produced by *Bacillus brevis* ATCC 8185 on an enzyme complex comprising three peptide synthetases, TycA, TycB, and TycC (tyrocidine synthetases 1, 2, and 3), via the nonribosomal pathway. However, previous molecular characterization of the tyrocidine synthetase-encoding operon was restricted to *tycA*, the gene that encodes the first one-module-bearing peptide synthetase. Here, we report the cloning and sequencing of the entire tyrocidine biosynthesis operon (39.5 kb) containing the *tycA*, *tycB*, and *tycC* genes. As deduced from the sequence data, TycB (404,562 Da) consists of three modules, including an epimerization domain, whereas TycC (723,577 Da) is composed of six modules and harbors a putative thioesterase domain at its C-terminal end. Each module incorporates one amino acid into the peptide product and can be further subdivided into domains responsible for substrate adenylation, thiolation, condensation, and epimerization (optional). We defined, cloned, and expressed in *Escherichia coli* five internal adenylation domains of TycB and TycC. Soluble His_6-tagged proteins, ranging from 536 to 559 amino acids, were affinity purified and found to be active by amino acid-dependent ATP-PP_i exchange assay. The detected amino acid specificities of the investigated domains manifested the colinear arrangement of the peptide product with the respective module in the corresponding peptide synthetases and explain the production of the four known naturally occurring tyrocidine variants. The K_m values of the investigated adenylation domains for their amino acid substrates were found to be comparable to those published for undissected wild-type enzymes. These findings strongly support the functional integrities of single domains within multifunctional peptide synthetases. Directly downstream of the 3′ end of the *tycC* gene, and probably transcribed in the tyrocidine operon, two tandem ABC transporters, which may be involved in conferring resistance against tyrocidine, and a putative thioesterase were found.

The biosynthetic system required for the production of the cyclic decapeptide tyrocidine is one of the prototypes for a protein template-driven pathway to synthesize biologically active peptides by the nonribosomal mechanism (20, 21, 30, 44, 46).

Large enzymes, called peptide synthetases, activate the amino acid constituents as aminoacyl adenylate at the expense of ATP and thioesterify them on the thiol moiety of an enzyme-attached cofactor, 4′-phosphopantetheine. Subsequently, activated amino acids are condensed stepwise in an amino- to carboxy-terminal direction. Previous investigations at the protein chemical level and, in particular, comparisons of several genes encoding peptide synthetases have revealed the modular architecture of this class of enzymes (4, 23, 28, 42, 44, 53). One module is defined to harbor all catalytic activities to incorporate a single amino acid residue into the peptide product. The modules coincide in number with the number of incorporated amino acids and are arranged in a colinear fashion, either within a single polypeptide chain, as is the case for all known fungal enzymes (15, 41, 54), or distributed among a set of enzymes, as is the case for bacterial peptide synthetases (20, 44).

On the basis of biochemical and sequence data, a module can be further subdivided into different domains (45, 53), each of which has a characteristic set of short sequence motifs (30, 44). The core of each module is the adenylation domain (about 550 amino acids [aa]), which bears the substrate recognition pocket and activates its cognate amino acid as adenylate at the expense of ATP. It belongs to the large family of adenylate-forming enzymes, such as firefly luciferase (2) and acyl coenzyme A synthetases (53). The peptidyl carrier domain (PCP), an analog to the acyl carrier protein of fatty acid and polyketide synthesis, is the site of attachment for the 4′-phosphopantetheine cofactor, which is covalently bound to the side chain of an invariant serine (24, 40, 44). Recently, the presence of such a cofactor at each module of a multimodular peptide synthetase has been shown, a finding that has established the multiple-carrier model for nonribosomal peptide synthesis (49). A putative condensation domain between each pair of adenylation domains of two consecutive modules was thought to catalyze peptide bond formation (5). Optional domains can also carry out modifications of the activated amino acid, like epimerization (45, 48) and N methylation (15, 54). In addition to the domains mentioned above, a putative thioesterase domain, which might be involved in product release and/or cyclization of the peptide chain, was found in bacterial systems as an integrated part of the modules incorporating the C-terminal amino acid.

It is one of the most prominent features of nonribosomal peptide synthesis that substrates are not restricted to the 20 proteinogenic amino acids; nonproteinogenic, hydroxy-, D-, and *N*-methylated amino acids can be incorporated into the products.

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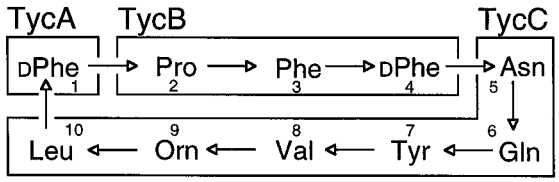
a)



b)

tyrocidine	Position [amino acid]			
	1	3	4	7
A	DPhe	Phe	DPhe	Tyr
B	DPhe	Trp	DPhe	Tyr
C	DPhe	Trp	DTrp	Tyr
D	DPhe	Trp	DTrp	Trp

FIG. 1. (a) Primary structure of tyrocidine A. Boxes indicate residues incorporated by the three tyrocidine synthetases, TycA, TycB, and TycC. Numbering is according to the order of synthesis (Orn, ornithine). (b) Amino acid substitutions in the four tyrocidines.

Tyrocidine is produced at the onset of the stationary phase of growth (10, 26); however, its actual function for the producer *Bacillus brevis* strain is unknown (17, 50), although several studies have suggested a regulatory role in the process of sporulation (27, 34, 39). The decapeptide has antibiotic activities against several gram-positive bacteria. Tyrocidine itself is in fact a mixture of four cyclic decapeptides, tyrocidines A, B, C, and D, in which Phe and Tyr residues are gradually replaced by Trp, depending on the relative concentrations of these amino acids in the growth medium (Fig. 1) (36). Each tyrocidine [tyrocidine A (D-Phe–Pro–Phe–D-Phe–Asn–Gln–Tyr–Val–Orn–Leu)$_{cyclic}$] contains two amino acids in D configuration and the nonproteinogenic residue L-ornithine. The synthesis of cyclic decapeptides is accomplished by tyrocidine synthetases TycA, TycB, and TycC, which activate one, three, and six amino acids, respectively (Fig. 1) (26). The *tycA* gene was the first of this class of enzymes to be cloned and sequenced (29, 55). Further sequence analysis has indicated that the other tyrocidine synthetases are organized in an operon, of which *tycA* and a part of the 5′ end of the putative *tycB* were sequenced (31). Since the tyrocidine synthetases are among the first and best investigated modular peptide synthetases, knowledge of the entire tyrocidine biosynthesis operon (*tyc* operon) was of fundamental interest.

Here, we report the cloning and sequencing of the entire *tyc* operon (39.5 kb) and demonstrate an approach for expressing active internal enzyme fragments. The specificities and activities of the recombinant proteins obtained are discussed in light of the general principles of nonribosomal peptide synthesis and particularly in terms of the implications of this on the synthesis of tyrocidine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. brevis* ATCC 8185 was maintained on agar plates of Difco Sporulation Medium (DSM) and grown in rich 2x YT medium at 37°C for DNA isolation procedures. *Escherichia coli* XL1 Blue (Stratagene, Heidelberg, Germany) was used for the preparation of recombinant plasmids. *E. coli* XL1 Blue P2 was used as the host for the λ EMBL3 genomic library and preparations of λ phages. Overexpression of recombinant proteins was carried out in *E. coli* M15 (Qiagen, Hilden, Germany).

Transformation of *E. coli* and DNA manipulations. Standard procedures were applied for the transformation of competent *E. coli* cells, preparation, digestion, and ligation of DNA fragments, and preparation of plasmid DNA (1, 37). The λ EMBL3 genomic library (Stratagene) was constructed by partial *Sau*3A digestion of chromosomal *B. brevis* DNA, salt density ultracentrifugation to exclude DNA fragments of <13 kb, and ligation into the *Bam*HI sites of the EMBL3 arms. In vitro packaging was performed with Gigapack III Gold (Stratagene) according to the manufacturer's protocol.

Cloning and sequencing of the entire *tyc* operon. To screen the genomic library of *B. brevis*, we used probes derived from the already known 5′ end of the *tyc* operon. A first screening was performed with probes made from the 1,586-bp *Ava*I fragment of plasmid pMS9 (31) and the 1,170-bp *Hin*dIII fragment from plasmid pTZ18UtycA (13) (probes I and II, respectively [Fig. 2]), yielding among others λ phage I.4. A second screening of the genomic library with probes constructed from the 351-bp *Sal*I and 1,816-bp *Hin*dIII/*Sal*I fragments of the 3′ end of λ I.4 (probes A and B, respectively [Fig. 2]) resulted in the isolation of λ A.7 and λ A.13. In total, ca. 6,000 plaques were screened, with approximately 500 plaques per agar plate (8.5-cm diameter). For plaque transfer, plates were prechilled to 4°C, covered with a positively charged nylon membrane (colony-plaque screen; Du Pont), and left for 15 min at 4°C. Membranes were carefully taken off and laid upside down on fresh agar plates. After incubation overnight at 37°C, membranes were incubated for 5 min on Whatman paper soaked with Southern I solution (43) and twice for 5 min on Whatman paper soaked with Southern II solution (43), immediately followed by UV cross-linking with a UV Stratalinker 1800 (Stratagene). Cell debris was carefully wiped off after membranes had been washed for 60 min at 65°C in 15 mM sodium chloride–1.5 mM sodium citrate (pH 7.0)–0.1% (wt/vol) sodium dodecyl sulfate (SDS). After this procedure, membranes were prepared for hybridization with fluorescein-labeled probes and the ECL random prime labeling and detection system (Amersham/Buchler, Braunschweig, Germany) according to the manufacturer's manual. Plaques hybridizing with one of the probes were isolated from the original agar plate and purified to homogeneity by repeated rounds of hybridization with the same probe.

The insert DNA of λ phage I.4 was mapped and subcloned in pBluescript SK (−) with *Sal*I. *Hin*dIII was used to subclone λ A.7 and λ A.13 into pBluescript SK (−). Subclones of >3 kb were occasionally further subcloned with *Hin*dIII, *Hin*cII, and *Pst*I. The sequencing of double-stranded pBluescript plasmids was performed with standard universal and SK primers and oligonucleotides derived from already determined sequences (all primers used in this study were synthesized by MWG Biotech, Ebersberg, Germany). Small gaps between subclones were filled by PCR amplification and sequencing of the product or by direct sequencing of the λ DNA template. Reactions were carried out by the chain termination method (38) with dye-labeled dideoxy terminators from a PRISM ready reaction dyedeoxy terminator cycle sequencing kit with AmpliTaq FS polymerase (Applied Biosystems) according to the manufacturer's protocol and analyzed on an ABI 310 genetic analyzer.

Amplification and cloning of internal gene fragments corresponding to adenylation domains. The pQE60-pREP4 overexpression system (purchased from Qiagen) (45) was used to clone PCR products in which *Nco*I and *Bam*HI sites were introduced by PCR with the following 5′-modified primers (restriction sites are underlined, and modified sequences are in italics): TycB1 (ProA2), 5′-ProA-*Nco*I (5′-*TATCCATGG*TTCCGTTGCTGACAGAAG-3′) and 3′-ProCA-*Bam*HI (5′-*ATAGGATCC*CAAGATTTGCTCCCAAATCGC-3′); TycB3 (PheA4), 5′-PheA3-*Nco*I (5′-*TTACCATGG*TGACAGCAGCAGAAAAGC-3′) and 3′-PheA3-*Bam*HI (5′-*ATTGGATCC*CAGCTCGGTTTGCGG-3′); TycC1 (AsnA5), 5′-AsnA-*Nco*I (5′-*ATACCATGG*AGATCGGCATCATCACC-3′) and 3′-AsnA-*Bam*HI (5′-*TATGGATCC*CTCGATTTCATTTTGCGG-3′); TycC2 (GlnA6), 5′-GlnA-*Nco*I (5′-*TATCCATGG*TGACGGAAGCGGAAAAAC-3′) and 3′-GlnA-*Bam*HI (5′-*TATGGATCC*CTTGGCTTCCCATTCGTTG-3′); and TycC3 (TyrA7), 5′-TyrA-*Nco*I (5′-*ATACCATGG*CGTTGTCCGAGATCACCA TG-3′) and 3′-TyrA-*Bam*HI (5′-*TATGGATCC*CACCGCATTTGTCGGCG-3′). PCR from chromosomal *B. brevis* DNA was carried out with the Expand long-range PCR system (Boehringer, Mannheim, Germany) as described in the manufacturer's manual. Typically, annealing was performed at 54°C for 30 s for the first 5 cycles and at 62°C for 30 s for the next 30 cycles. PCR products were purified with a QIAquick-spin PCR purification kit (Qiagen), digested with *Nco*I and *Bam*HI, and cloned into the digested pQE60 vector. Cloning into the *Bam*HI site of pQE60 results in appending the amino acid sequence GSRSHHHHHH at the C terminus of the recombinant protein.

Overexpression and purification of internal adenylation domains. pQE60 plasmids containing PCR-amplified gene fragments were transformed in *E. coli* M15(pREP4) and expressed in 2x YT medium at 30°C as previously described (45), except that cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an A_{600} of 0.7 to 0.9 and allowed to grow for an additional 2 h before being harvested. Purification of the His_6-tagged proteins (in order), ProA2, PheA4, AsnA5, GlnA6, and TyrA7 (named according to the primary structure of tyrocidine A [Fig. 1 and 2]), was basically carried out as previously described (45) by Ni^{2+}-affinity chromotography, but cells were broken by three passages through a French press. As judged from Coomassie blue-stained SDS polyacrylamide gels, the proteins were purified to apparent homogeneity after a

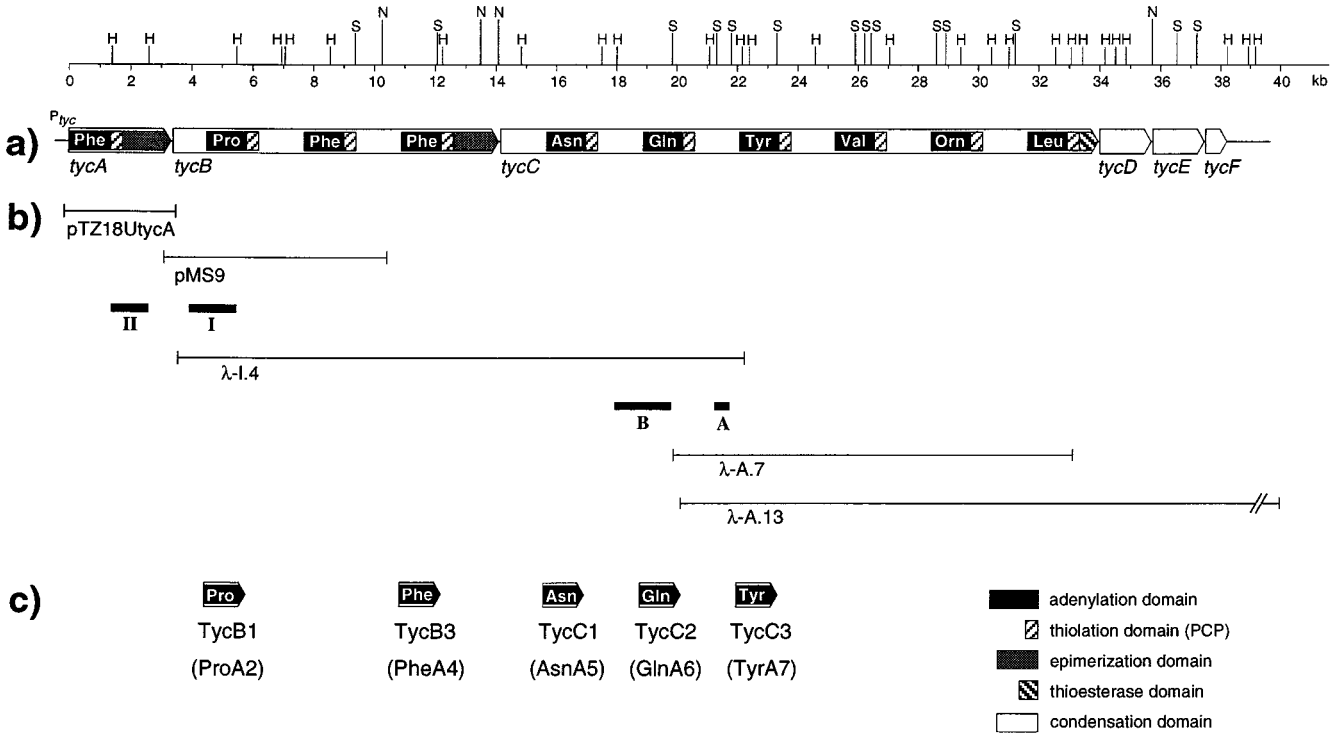


FIG. 2. Cloning, sequencing, and biochemical characterization of the tyrocidine biosynthesis operon. (a) The entire tyrocidine operon comprising the genes from *tycA* to *tycF* is shown. The domain organizations of the peptide synthetases deduced from *tycA*, *tycB*, and *tycC* are illustrated within the genes (abbreviations used in the restriction map: H, *Hin*dIII; S, *Sal*I; and N, *Nco*I). (b) Clones of the previously sequenced 5′ end of the operon, probes used for screening the λ EMBL3 genomic library, and λ phages isolated in this study. (c) Gene fragments corresponding to adenylation domains that were overexpressed and biochemically analyzed.

single chromatography step and the results were in good agreement with the calculated sizes (ProA2, 559 aa and 63.0 kDa; PheA4, 536 aa and 60.0 kDa; AsnA5, 546 aa and 60.8 kDa; GlnA6, 545 aa and 60.4 kDa; and TyrA7, 550 aa and 60.3 kDa) (see Fig. 3). Protein concentrations were determined by using the calculated molar extinction coefficients for the A_{280} (ProA2, 37,580 M^{-1} cm^{-1}; PheA4, 58,370 M^{-1} cm^{-1}; AsnA5, 53,150 M^{-1} cm^{-1}; GlnA6, 68,020 M^{-1} cm^{-1}; and TyrA7, 54,770 M^{-1} cm^{-1}).

ATP-PP_i exchange. This assay was performed in two ways. First, in order to examine substrate specificity under saturated conditions, it was carried out with all constituent amino acids of tyrocidine (including D-Phe, L-Trp, and a control without amino acid). Second, it was performed to determine the apparent K_m values for ATP and the amino acid under investigation with varied substrate concentrations from 0.02 to 0.20 mM. The experimental procedure was previously reported (45). The enzyme concentrations were 0.3 (TyrA7 and PheA4), 0.6 (ProA2), and 3 (AsnA5 and GlnA6) μmol/liter. Reactions were started with the addition of enzymes and incubated at 37°C for 15 min at pH 7.1.

Nucleotide sequence accession number. The nucleotide sequences from *B. brevis* ATCC 8185 described here have been submitted to GenBank under accession no. AF004835.

RESULTS

Cloning and sequencing of the *tyc* operon. The 5′ end of the *tyc* operon (5,007 bp) was previously cloned and sequenced (31, 55). This part included the promoter region, the entire *tycA* gene, and 735 bp of the *tycB* gene. DNA rearrangements have subsequently been found in the λ phage, EMBL25-1, used in those studies; this does not permit its utilization for further investigations. In order to clone the rest of the *tyc* operon, we therefore constructed a new λ EMBL3 genomic library of *B. brevis* ATCC 8185 and screened it with DNA probes derived from the known parts of the *tycA* and *tycB* genes. Of approximately 6,000 plaques screened, 5 λ phages that hybridized with the downstream *tycB* probe but not with the upstream *tycA* probe (probes I and II [Fig. 2]) were isolated; therefore, the cloned part of the *tyc* operon should have been extended in a downstream direction. λ phage I.4 (18,555-bp insert DNA) was mapped, and all fragments obtained by *Sal*I digestion were subcloned into pBluescript SK (−). Two DNA fragments of the 3′ part of the λ I.4 insert were used for a second screening of the genomic library, yielding four λ phage clones selected again for hybridization with the downstream probe but not with the upstream probe (probes A and B [Fig. 2]). Of these, λ phages A.7 (ca. 15-kb insert DNA) and A.13 (ca. 22 kb) were subcloned into pBluescript SK (−) with *Hin*dIII. In total, the isolated λ phages I.4 and A.13 extend the previously cloned part of the DNA locus containing the entire *tyc* operon to more than 44 kb (Fig. 2).

The pBluescript SK (−) subclones containing *Sal*I and *Hin*dIII fragments of λ I.4, λ A.7, and λ A.13 were sequenced as described in Materials and Methods. The nucleotide sequence was read over all restriction sites by sequencing either the λ DNA template or an appropriate PCR product. Finally, with synthesized oligonucleotides, the opposite strand of the entire *tyc* operon was sequenced. The previously reported *tycA* and the 5′ end of *tycB* were resequenced, resulting in a few corrections.

The overall G+C content of the nucleotide sequence determined for the *tyc* operon is almost 54% and reasonably higher than those reported for other bacilli.

The *tyc* operon contains the genes, *tycA*, *tycB*, and *tycC*, that encode the three tyrocidine synthetases. Sequence analysis of the region downstream of the previously described *tycA* gene, encoding the starter peptide synthetase TycA, revealed the presence of two large open reading frames (ORFs) transcribed in the same direction. The first ORF (10,764 bp), *tycB*, is located 95 bp downstream of the TAA stop codon of *tycA* and starts with an ATG codon 7 bp upstream of which a putative

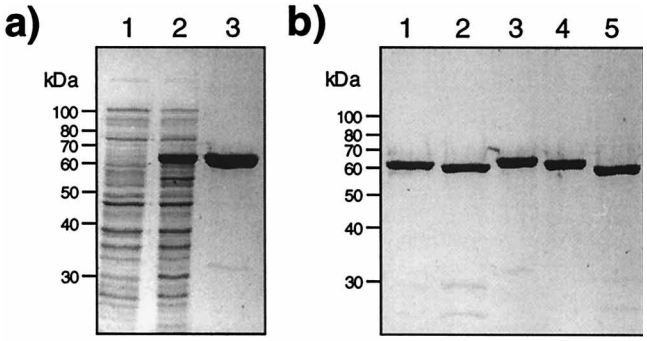


FIG. 3. SDS-polyacrylamide gels of adenylation domains overexpressed in *E. coli*. (a) Example of overexpression of adenylation domain AsnA5. Lanes: 1, whole-cell extract before induction; 2, whole-cell extract after induction with IPTG for 2 h; 3, purified protein. (b) Purified adenylation domains used for biochemical analysis. Lanes: 1, ProA2; 2, PheA4; 3, AsnA5; 4, GlnA6; 5, TyrA7.

ribosome binding site (RBS) (CGAGG) can be found. Although 735 bp of this ORF has been previously reported (31), sequencing the opposite strand led to a few corrections. The gene product (404,562 Da) shows highest similarities (25 to 45% identity) to known peptide synthetases, namely, to GrsB (gramicidin S synthetase 2) (53). Three modules, each consisting of a putative condensation domain, an adenylation domain, and a thiolation domain, which is typical for peptide synthetases (44), were identified. The last module also contains at its C-terminal end the motifs that are typical for an epimerization domain (Fig. 2). These findings are in perfect agreement with the composition that was expected for TycB, which activates the second to fourth amino acids in tyrocidine (in tyrocidine A, Pro, Phe, and D-Phe [Fig. 1]) and epimerizes the last one (35).

The second ORF is located 105 bp downstream of the TGA stop codon of *tycB*, starting with an ATG codon that is preceded by 6 bp by a putative RBS (TGAGG). This ORF is 19,461 bp in size, encoding a gene product of 723,577 Da that was found to contain six modules. A putative thioesterase domain is located at the C-terminal end of the sixth module. This arrangement of domains fits the expectations for TycC, tyrocidine synthetase 3, which incorporates six amino acids (for tyrocidine A, Asn, Gln, Tyr, Val, Orn, and Leu) in their L configuration into the peptide product (35). We therefore designated this giant ORF *tycC* (Fig. 3), encoding the third and last tyrocidine synthetase, TycC.

From sequence comparisons, the gene products of the *tycB* and *tycC* genes can clearly be identified as peptide synthetases. The modules of tyrocidine synthetases have 34 to 60% identity with each other. The adenylation domains of the 10 modules are even better conserved, showing 40 to 63% identity. In comparisons to other peptide synthetases, we found particularly high similarities to the gramicidin S synthetases, GrsA and GrsB, of *B. brevis* ATCC 9999.

The especially high similarities to the gramicidin S synthetases were analyzed in more detail. The first adenylation domain of TycB and the last three adenylation domains of TycC exhibit particularly high sequence similarities (63 to 68% identity) to the four adenylation domains of GrsB. Such a high degree of similarity was already known for the two starter synthetases TycA and GrsA (62% identity). There are comparatively high identities for a few other pairs of adenylation domains within the tyrocidine synthetases: TycA and TycB3, 58%; TycB3 and TycC3, 63%; TycB3 and TycC10, 56%; and TycC3 and TycC10, 60%. The significance of these findings is discussed below in terms of amino acid specificity and evolutionary relationship.

The putative epimerization domains detected at the C termini of TycA and TycB are similar to the epimerization domains in GrsA (57 and 61% identities, respectively) and the surfactin synthetases SrfA-A and SrfA-B (mean, 48% identity). The domain localized at the C terminus of TycC, putatively designated a thioesterase domain, contains the motif GXSXG and reveals its highest similarity to the equivalent domain of GrsB (54% identity).

Two tandem ABC transporters and a thioesterase are located adjacent to the peptide synthetase genes. We detected downstream of the *tycC* gene three additional ORFs, *tycD*, *tycE*, and *tycF*, which are all transcribed in the same direction as are the tyrocidine synthetases (Fig. 3). The initiation codon ATG of *tycD* (1,788 bp) is located 85 bp downstream of the *tycC* stop codon and is preceded by 7 bp by a putative RBS (GAGGA). For *tycE*, we cannot identify an ATG start codon with a reasonable Shine-Dalgarno sequence. Initiation may occur from an ATG codon preceded by 7 bp by TGCGT, yielding an ORF of 1,752 bp situated 52 bp downstream of *tycD*. The deduced gene products, TycD (595 aa and 66,135 Da) and TycE (583 aa and 63,981 Da), are 36% identical. In data base searches, they revealed their highest similarities to members of the ABC transporter family. Hydrophilicity plots of TycD and TycE showed the presence of six transmembrane helices in the N-terminal part of each protein (not shown). The C-terminal part of each protein contains the ATP-binding cassette. The *tycF* ORF (735 bp) is 59 bp from *tycE* and is read in the same direction. A potential RBS (GGGAG) is 7 bp upstream of the ATG initiation codon. The gene product TycF (244 aa and 27,773 Da) shows sequence similarities to thioesterases and contains the conserved motif GXSXG. The highest similarity for TycF is found with GrsT, the first gene of the gramicidin S biosynthesis operon (35% identity) (22). We therefore anticipate that *tycF* codes for the thioesterase of uncertain function that always seems to be associated with bacterial operons which encode peptide synthetases.

Operon organization. *tycB* has previously been reported to be part of an operon with *tycA* (31). The distances between the genes reported here (*tycA*, *tycB*, *tycC*, *tycD*, *tycE*, and *tycF*) range from 52 to 105 bp. In other bacterial systems, like gramicidin S (22) and surfactin (4), the genes encoding thioesterases, GrsT and SrfA-TE, respectively, have been shown to be organized in an operon with the genes encoding peptide synthetases. We therefore assume that *tycF*, encoding the thioesterase, is located within the *tyc* operon, as are the *tycD* and *tycE* genes, although we cannot exclude the presence of additional promoters or termination signals. We could not identify a reasonable termination loop between any of the genes from *tycA* to *tycF* or downstream of *tycF*. Sequence analysis of the region ca. 1.8 kb downstream of *tycF* did not reveal significant similarities to known proteins. In particular, we have not yet found a gene encoding a 4′-phosphopantetheinyl transferase, whose presence in the vicinity of the *tyc* operon is expected (24). Further sequencing is in progress.

Amplification, expression, and biochemical investigation of internal adenylation domains of TycB and TycC. DNA fragments coding for the adenylation domains of modules TycB1 (ProA2), TycB3 (PheA4), TycC1 (AsnA5), TycC2 (GlnA6), and TycC3 (TyrA7) (Fig. 2) were amplified from chromosomal *B. brevis* DNA and cloned into IPTG-inducible expression vectors as described in Materials and Methods. The constructs were confirmed by sequencing fusion sites. The enzyme fragments were overexpressed in *E. coli* as His_6-tagged proteins and purified by Ni^{2+}-affinity chromatography. Figure 3 shows the typical expression patterns of one adenylation domain (AsnA5) and five purified proteins. All proteins were found

virtually completely in the soluble fraction after French press lysis of *E. coli* cells, as judged from Coomassie blue-stained SDS-polyacrylamide gels. Their observed sizes on SDS-polyacrylamide gels (Fig. 3) are in good agreement with the calculated molecular masses of 60.0 to 63.0 kDa. The purified adenylation domains were biochemically investigated with respect to their activity in the ATP-PP_i-exchange reaction. First, in order to determine amino acid specificity, each protein was incubated with all constituent amino acids of tyrocidine, including L-Trp, D-Phe, and a control without amino acid. The five adenylation domains were found to activate the amino acids corresponding to their positions within the multifunctional synthetases TycB and TycC (Fig. 4), given the colinearity of the modular arrangement with the primary structure of the peptide product. ProA2 exclusively activated L-Pro. PheA4 activated L-Trp (100%), L-Phe (48%), and D-Phe (16%). (The highest activity was set at 100%. The background and controls were between 0.1 and 1%.) AsnA5, the first adenylation domain of TycC, exclusively activated L-Asn, whereas GlnA6 activated L-Gln (100%) and slightly activated L-Val and L-Leu (1 to 2%). TyrA7 activated L-Trp (100%) and L-Tyr (78%) and slightly activated L-Leu and L-Phe (1 to 2%). The very high specificities of PheA3 and TyrA7 for L-Trp reveal that they are actually tryptophan-activating domains. This observation is in accordance with findings that the corresponding position in tyrocidine can be replaced by L-Trp, resulting in a product mixture of tyrocidines A, B, C, and D (36) (Fig. 1) (see Discussion). After having qualitatively confirmed the enzymatic properties of the single domains, we determined the K_m values of the recombinant adenylation domains for their cognate amino acid(s) and for ATP (Table 1). The determined K_m values ranged from 0.08 to 0.85 mM for the amino acid substrates and from 0.6 to 1.9 mM for ATP. They are comparable to those determined for wild-type GrsB, the most closely related peptide synthetase (18). The K_m values obtained are also in the same order of magnitude as those reported for other peptide synthetases (8, 33, 45) and for aminoacyl-tRNA synthetases. The PheA4 and TyrA7 domains did indeed have higher affinities to L-Trp (K_m values of 0.08 and 0.10 mM, respectively) than to L-Phe (0.45 mM) and L-Tyr (0.30 mM), respectively. Turnover rates were not determined precisely; however, an extrapolation of the data obtained for the determination of K_m values revealed specific activities similar to those of wild-type GrsA (45) and TycA (13) (not shown).

Our results prove that the five internal enzyme fragments investigated indeed exhibit the enzymatic properties of adenylation domains. Their functional integrities, as demonstrated by determinations of substrate specificities and K_m values, strongly support the view that these domains work independently within peptide synthetases and act in concert with other domains in an ordered manner during the peptide elongation reaction.

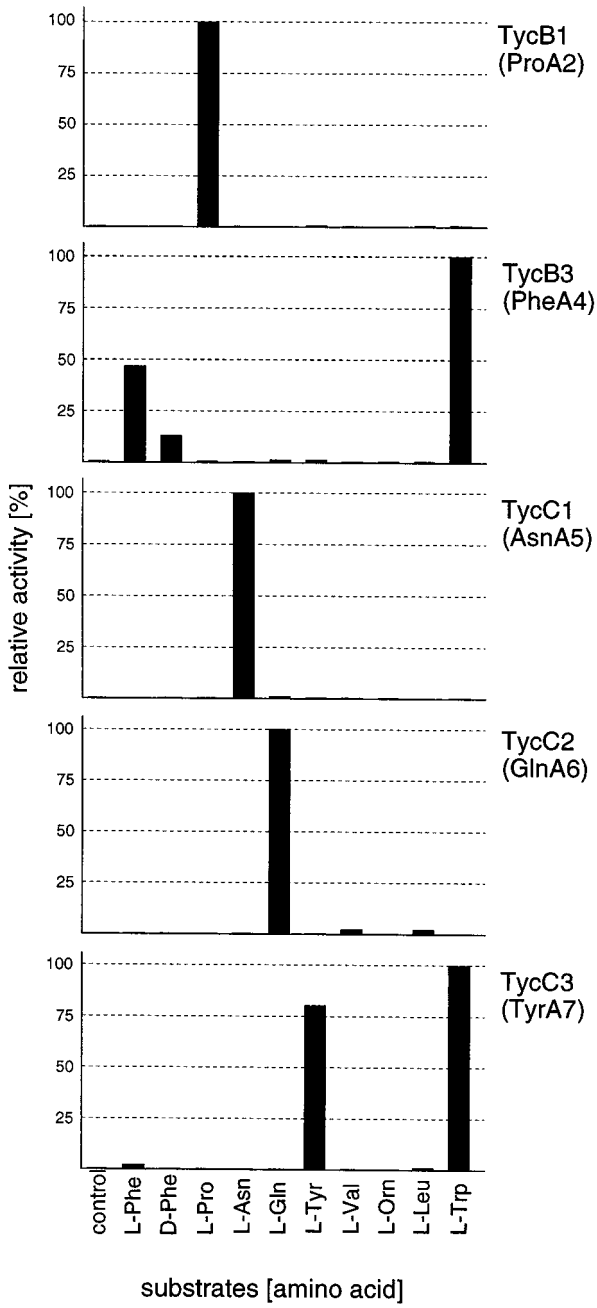


FIG. 4. Relative substrate specificities of internal adenylation domains. Internal adenylation domains were investigated in terms of activity in the ATP-PP_i exchange reaction with the amino acids of tyrocidine and a control without amino acid. The highest activities were set at 100%. The background was below 1%. The specificities of the different domains coincide with the primary structures of the tyrocidines.

DISCUSSION

We cloned and sequenced a DNA locus of *B. brevis* of about 41 kb which contains the entire tyrocidine biosynthesis operon (*tyc* operon) (Fig. 2). We identified downstream from the previously reported *tycA* gene (55), and obviously transcribed in the operon (31), the genes, *tycB* and *tycC*, encoding tyrocidine synthetases TycB and TycC, respectively (tyrocidine synthetases 2 and 3). In agreement with the protein chemical findings, *tycB* codes for a three-module-bearing enzyme, including an epimerization domain associated with the last module, and *tycC* codes for a six-module multienzyme. The compositions of the tyrocidine synthetases therefore seem to obey the empirical rules for the modular architecture of peptide synthetases, which have been elaborated in recent years by sequencing several such genes (21, 30, 44). TycC (723,577 Da), which activates six amino acids, is the largest reported bacterial peptide synthetase.

We were particularly interested in comparing the tyrocidine and gramicidin S synthetases. Both antibiotics are produced from *B. brevis* strains and are structurally closely related. Gramicidin S is a cyclic dimer of one-half of the tyrocidine

TABLE 1. K_m values of internal adenylation domains

Adenylation domain	Substrate (amino acid)	K_m (mM)	
		Amino acid	ATP
ProA2	L-Pro	0.35 ± 0.10	0.9 ± 0.3
PheA4	L-Phe	0.45 ± 0.10	1.2 ± 0.3
	L-Trp	0.08 ± 0.05	1.0 ± 0.3
AsnA5	L-Asn	0.85 ± 0.20	1.7 ± 0.3
GlnA6	L-Gln	0.70 ± 0.20	1.9 ± 0.3
TyrA7	L-Tyr	0.30 ± 0.10	1.0 ± 0.3
	L-Trp	0.10 ± 0.05	0.6 ± 0.2

sequence (Val–Orn–Leu–D-Phe–Pro). The two gramicidin S synthetases, GrsA and GrsB, consist of only five modules each (22, 53) and dimerize by an as-yet-unknown mechanism the linear intermediates (D-Phe–Pro–Val–Orn–Leu) to the cyclic decapeptide (20). In tyrocidine synthesis, the sequence Phe–D-Phe–Asn–Gln–Tyr is inserted between the Pro and Val residues (Fig. 1). This analogy between the chemical structures of the products has now been confirmed on the molecular level of the synthetases. The corresponding modules of the two systems show significantly higher similarities to each other than they do to other modules of peptide synthetases. These findings suggest that the tyrocidine synthetases have evolved by the insertion of five modules (the last two of TycB and the first three of TycC) from the gramicidin S synthetases or that the gramicidin S synthetases have evolved by the deletion of five domains of the tyrocidine system. Strikingly, however, the DNA locus that includes the *grs* operon has a G+C content of 36% (22, 53), whereas the *tyc* operon has a remarkably high G+C content of almost 54% (the G+C contents reported for single genes from several *B. brevis* strains have ranged between 36 and 53%).

A comparison of the highly similar *tyc* and *grs* sequences did not reveal significant differences that could clearly be attributed to the ability of GrsB to dimerize (either intra- or intermolecularly) two pentapeptide intermediates. At its C terminus, TycC also contains a putative thioesterase domain of about 250 aa that might be responsible for product release and/or cyclization. This domain is 54% identical to the respective C-terminal GrsB domain. However, when they were aligned, we found a 26-aa insertion between the peptidyl carrier domain of the last GrsB module and the putative thioesterase domain (QPILVNVEADREALSLNGEKQRKNIE). Whether this region contributes to the dimerization activity of GrsB remains a matter of speculation.

In order to biochemically characterize and to gain insight into the modular compositions of the tyrocidine synthetases, we heterologously expressed internal enzyme fragments corresponding to the adenylation domains. Numerous attempts to achieve this goal had previously been undertaken in our and other laboratories. For example, the first adenylation domain of TycB was expressed at high levels in *E. coli* but found to be completely insoluble and inactive (7). However, defining the right N terminus of the construct appeared to be the critical parameter. When the N terminus of the internal adenylation domain chosen was 95 to 100 aa in front of motif 1 (LKAGGA) and the C terminus was about 100 aa behind motif 5 (RIELG EIE), two core motifs highly conserved among peptide synthetases (44), we were able to express soluble proteins. Here, we describe the expression of five internal enzyme fragments of TycB and TycC, corresponding to the minimal size of adenylation domains (536 to 559 aa and 60 to 63 kDa) (Fig. 2 and 3). These proteins were found to be virtually completely soluble when they were expressed in *E. coli* under the conditions used (Fig. 3a). They were enzymatically active in the ATP-PP_i exchange reaction with clear substrate specificities (Fig. 4). The determined K_m values of 0.08 to 0.85 mM for the cognate amino acid(s) and 0.6 to 1.9 mM for ATP (Table 1) are comparable to values reported for wild-type GrsB (18), the most similar peptide synthetase. These findings have important implications on our understanding of the overall architecture of peptide synthetases. The feasibility of expressing protein fragments taken out of a multifunctional enzyme without changing their catalytic properties strongly supports the view of each peptide synthetase as a line of several domains that act in concert only according to their order within this line. Our results presented here are the most straightforward support for the modular architecture of peptide synthetases, which has previously been suggested from sequence analysis and module swaps (47).

The expression of recombinant adenylation domains of peptide synthetases has been the goal of much effort in our and other laboratories, since these domains harbor the substrate specificity and thereby dictate the primary structure of the final product. Although heterologous expression of peptide synthetase fragments has previously been described by a few authors, in those cases, larger proteins corresponding mostly to an entire module (110 to 140 kDa) were examined (8, 14, 16, 23, 24). C-terminal-truncated GrsA and TycA derivatives are the only reported examples of recombinant minimal adenylation domains expressed in a heterologous host, but they retained their native N termini and were constructed from relatively simple peptide synthetases (comprising only one module) (6, 45).

In our laboratory, this method has successfully been adopted to domains of other *Bacillus* systems. It may prove to be a useful tool in understanding the primary structures of peptides produced by gene clusters of as-yet-unknown function, such as results from the genome sequencing project of *Bacillus subtilis* (52). It is also of great interest for crystallographic studies, since such proteins should be as small as possible. Very recently, the corresponding adenylation domain of GrsA has been crystallized (3).

The observed specificities of the five adenylation domains tested shed light on an interesting aspect of nonribosomal synthesis. For several systems, like tyrocidine (9, 36), bacitracin (51), surfactin (12), cyclosporin (19, 25), and enniatin (32), it has previously been shown that nutrient conditions can affect the constitution of the peptide product, either in vivo by adding certain amino acids to the medium or in vitro in cell-free systems with arbitrarily chosen substrate concentrations. Actually, most nonribosomally produced peptides are a mixture of at least two compounds. The relaxed specificities of peptide synthetases compared to those of aminoacyl-tRNA synthetases are a known feature of nonribosomal peptide synthesis that may be explained by the much higher evolutionary pressure put on the latter.

In the case of tyrocidine, four products with amino acid substitutions can be isolated from growing cells (Fig. 1) (36). Tyrocidine A has Phe residues at positions 1, 3, and 4 and a Tyr residue at position 7. In tyrocidines B, C, and D, the residues at positions 3, 4, and 7 are gradually replaced by Trp, whereas position 1 remains unchanged (Fig. 1). The relative amounts of the four tyrocidines can be shifted by either adding Phe or Trp to the growth medium (36). We investigated the adenylation domains corresponding to positions 4 and 7 and traced the variabilities of the products back to substrate recognition in the activation reaction (Fig. 4). The adenylation domain of PheA4 (position 4) indeed activated L-Phe and L-Trp; TyrA7 (position 7) activated L-Tyr and L-Trp. We assume that the domain of TycB2 (position 3), which was not examined in our studies, also

activates L-Phe and L-Trp. On the other hand, the adenylation domain of TycA (position 1) was investigated by Dieckmann et al. and found to activate L-Phe but not L-Trp (6), in agreement with feeding studies that show this position is invariant. Taken together, the adenylation domains of the tyrocidine synthetases exemplify a remarkable variability of specificity in nonribosomal peptide synthesis. Either domains can accurately distinguish between two structurally related substrates, as we have demonstrated for the Asn- and Gln-activating domains and for the Phe- and Tyr-activating domains (Fig. 4), or domains can exhibit relaxed but defined specificities for two amino acids, as found for PheA4 and TyrA7, both of which also activated Trp. It is therefore tempting to suppose that the relaxed specificities of some domains originate from biological requirements. These can of course be speculative only, as is the function of tyrocidine itself. A higher or broader antibiotic activity of a mixture of products seems reasonable, but precise adjustment of a messenger to regulate the processes that lead to sporulation is also conceivable. A highly similar adenylation domain from gramicidin S synthetase GrsA (45), which does not activate L-Trp, has very recently been crystallized with the substrate L-Phe (3). The residues forming the hydrophobic binding pocket for the side chain of L-Phe are identical to those of TycA, slightly different for the adenylation domains of TycB2 and PheA3, and even more altered toward hydrophilic residues in the case of TyrA7. A great deal of the poorly understood specificity code of peptide synthetases could be discovered by mutational analysis of the discussed proteins.

Located directly downstream of the tyrocidine synthetases, two ABC transporters (TycD and TycE), which are 38% identical, and a putative thioesterase (TycF), which is 34% identical to GrsT of the gramicidin S biosynthesis operon (Fig. 2) (22), were detected. The function of the thioesterase, seemingly belonging to all bacterial clusters for nonribosomal peptide synthesis, is unknown. The locations of the two tandem transporters within the operon suggest a role in conferring resistance on the producer cell against tyrocidine via secretion. Two such tandem ABC transporters have recently been found in a locus of *Proteus mirabilis* that encodes peptide/polyketide synthetases (11). Disruption of one of the transporters or of the synthetase resulted in the same phenotype as that of a swarming-defective strain, suggesting involvement in product secretion (11). Work is in progress to clarify the potential role of the ABC transporters TycD and TycE in the secretion of tyrocidine.

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