JOURNAL OF BACTERIOLOGY, May 1994, p. 2654–2662
0021-9193/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 176, No. 9

Analysis of Core Sequences in the D-Phe Activating Domain of the Multifunctional Peptide Synthetase TycA by Site-Directed Mutagenesis

MARTIN GOCHT AND MOHAMED A. MARAHIEL*

Fachbereich Chemie/Biochemie, Philipps-Universität Marburg, D-35032 Marburg, Germany

Received 17 November 1993/Accepted 22 February 1994

The D-phenylalanine-activating enzyme tyrocidine synthetase I (TycA) from *Bacillus brevis* ATCC 8185 was overexpressed in *Escherichia coli*, purified to homogeneity, and assayed for ATP-PP_i exchange and covalent binding of phenylalanine by the thiotemplate mechanism. Amino acid exchanges in four different cores of TycA created by site-directed mutagenesis revealed the amino acid residues involved in aminoacyladenylate formation and in covalent thioester formation. Mutations in the putative ATP-binding site SGTTGKPKG caused a decreased phenylalanine-dependent ATP-PP_i exchange activity to 10% of the wild-type level for a Lys-186-to-Arg substitution and an almost complete loss of activity (<1%) for a Lys-186-to-Thr exchange. Alteration of Asp-401 to Asn in the ATPase motif TGDL of TycA decreased the phenylalanine-dependent ATP-PP_i exchange activity to 75% of wild type, while an Asp-401-to-Ser mutation decreased the activity to 10% of the wild-type level. Replacement of Ser-562 in the putative thioester-binding motif LGGDSI to Ala or Gly caused a reduction in trichloroacetic acid-precipitable TycA-[^{14}C]phenylalanine complex to one-third of the wild-type level. However, no cleavable [^{14}C]phenylalanine could be detected after treatment with performic acid, indicating that the resulting mutant was unable to form thioester with phenylalanine. In *E. coli*, TycA was labeled with β-[^{3}H]alanine, a precursor of 4′-phosphopantetheine, indicating that TycA is modified with a β-alanine-containing cofactor.

Under sporulation-inducing growth conditions, *Bacillus brevis* ATCC 8185 produces the peptide antibiotics tyrocidine and linear gramicidin (18). Both compounds are produced nonribosomally by large multifunctional enzymes via the protein thiotemplate mechanism (32). The enzymes involved in the biosynthesis of linear gramicidin have not been completely characterized (2, 23), but the biosynthesis of tyrocidine has been studied in detail (26, 27, 29, 30, 49). The cyclic decapeptide tyrocidine (D-Phe–Pro–Phe–D-Phe–Asn–Glu–Tyr–Val–Orn–Leu-$)_c$ is synthesized by the aid of three multifunctional enzymes; tyrocidine synthetases 1 (TycA), 2 (TycB), and 3 (TycC) activate and polymerize the 10 substrate amino acids.

TycA initiates peptide synthesis by activation of L-phenylalanine to the aminoacyladenylate by using ATP as the AMP donor. The activated phenylalanyladenylate is then covalently bound to TycA by a thioester linkage. After racemization, the D-isomer of phenylalanine is transferred from TycA to an activated proline residue on TycB. TycB activates proline and links it to two molecules of phenylalanine, while TycC, the third multienzyme, is responsible for the activation and condensation of the remaining six amino acids. Successive transpeptidation is achieved with the aid of the cofactor 4′-phosphopantetheine, which acts as an internal transport system for the growing peptide chain. To date, the presence of 4′-phosphopantetheine as a cofactor has been demonstrated for TycB and TycC (28, 30) but not for TycA. The cloning (34) and sequencing (64) of the *tycA* gene, however, revealed a putative 4′-phosphopantetheine-binding site similar to those found in *grsB* (58) and *srfA* (7, 12).

* Corresponding author. Mailing address: Fachbereich Chemie/Biochemie, Philipps-Universität Marburg, Hans-Meerweinstrasse, D-35032 Marburg, Germany. Phone: (49) 6421-285722. Fax: (49) 6421-282191. Electronic mail address: marahiel@ps1515.chemie.uni-marburg.de.

The *tycA* gene is located at the 5′ end of the *tyc* operon (39), which is transcriptionally induced when cells enter the stationary phase of growth. Studies on the *tyc* promoter, which is located 330 bp upstream of the *tycA* initiation codon, revealed its dependence on the Spo0A-AbrB regulatory system (13, 35, 36, 46). The nucleotide sequence of *tycA* codes for a protein of 1,087 residues with a calculated mass of 126 kDa.

Sequence comparisons of TycA with other peptide synthetases and with a family of carboxy acid-activating enzymes revealed a high degree of similarity within a region of about 600 amino acids, which was designated the functional domain (58). This domain contains highly conserved core sequences of 3 to 10 amino acid residues which are believed to be essential for amino acid activation and thioester formation (33). Figure 1 shows the functional domain of TycA with the relative locations and sequences of six cores. From sequence homology, cores 2 and 4, SGTTGKPKG and TGD, are believed to be involved in ATP binding and hydrolysis (58), whereas the serine of core 6, LGGHSL, has been shown to be the site of thioester formation in the GrsB-Val and GrsB-Leu domains, the second and fourth domains in gramicidin S synthetase 2 (54). Furthermore, the serine of this core in the first to fourth domain of the surfactin-synthesizing enzymes is indispensable for the biosynthesis of surfactin and thioester formation (11, 61, 62). Core 6 is present only in the thioester-forming enzymes, not in the adenylate-forming enzymes. It also shows good homology to the sequence motif of the 4′-phosphopantetheine-binding sites of acyl carrier proteins found in fatty acid and polyketide synthetases (54). The sequence of core 3, GELCIGG, contains the only cysteine which is conserved among different multienzymes, including TycA, GrsB-Val, GrsB-Orn, GrsB-Leu (the second to fourth domain of gramicidin S synthetase 2) (58), GrsA (gramicidin S synthetase 1) (17), luciferase (10), and 4-coumarate:coenzyme A ligase (32).

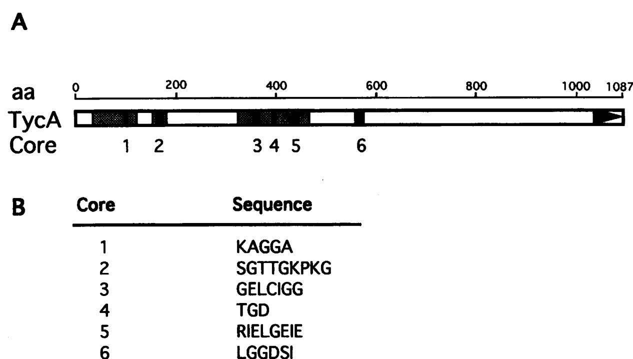

FIG. 1. (A) Organization of cores in TycA. Solid bars represent sequences with strong homologies to other peptide synthetases. Shaded bars represent sequences with reduced homologies. aa, amino acid. (B) Amino acid sequence of cores 1 to 6 in the one-letter code.

This cysteine thus represents a possible candidate for a direct thioester linkage.

The aim of the present work was to obtain solid evidence for the participation of multienzyme core sequences in substrate amino acid activation and thioester formation. This was achieved by site-directed mutagenesis of TycA and subsequent analysis of mutants. A simple test for phenylalanine racemization is also presented, and the labeling of TycA with β-[^{3}H]alanine shows the incorporation of a 4′-phosphopantetheine precursor in TycA.

MATERIALS AND METHODS

Bacterial strains and media. Subcloning and expression of TycA were done in *Escherichia coli* JM105 (38). Transformation of mutated plasmids during site-directed mutagenesis was performed in *E. coli* TG1 (37). Incorporation experiments with β-[^{3}H]alanine were done in *E. coli* SJ16 (*panD*), a β-alanine auxotroph (19), and in *E. coli* JM105. Medium 2×YT (6) was used as the rich medium for *E. coli.*

Materials. Tetrasodium $^{32}PP_i$ (16.06 Ci/mmol) and β-[3-^{3}H]alanine (91.5 Ci/mmol) were purchased from Du Pont-New England Nuclear (Bad Homburg, Germany). L-[U-^{14}C]phenylalanine (474 mCi/mmol) and [α-^{35}S]dATP (1,000 Ci/mmol) were purchased from Amersham/Buchler (Braunschweig, Germany). Superdex 200 HR 16/60, Phenylsepharose HR 16/10, and Mono Q HR 5/5 were run on a fast protein liquid chromatography (FPLC) system from Pharmacia LKB Biotechnology Inc. (Freiburg, Germany).

Cloning and mutagenesis. Subcloning of the 3.6-kb *Bam*HI-*Pst*I fragment of pGC12 (36) containing the structural gene of *tycA* and an 80-bp upstream region into the multiple cloning site of pTZ19U (Pharmacia) was performed by standard procedures (51). Single-amino-acid exchanges were carried out by site-directed mutagenesis by the method of Taylor et al. (57) with a kit from Amersham/Buchler (Braunschweig, Germany). The oligonucleotides used for amino acid substitution were obtained from TIB Molbiol (Berlin, Germany) and M. Krause (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany). The sequences of the oligonucleotides were as follows: oligo G180A, 5′-GCCTGTCGTGGCTGAGGT GTA-3′; oligo G183A, 5′-CTTTTGGCTTGGCTGTCGTG C-3′; oligo P185V, 5′-GGTGCCTTTTACCTTGCCTGT-3′; oligo K186R, 5′-GCATGGTGCCTCTTGGCTTGCCTGTC G-3′; oligo K186T, 5′-GCATGGTGCCTGTTGGCTTGCCT GTC-3′; oligo G187A, 5′-CAAGCATGGTGGCTTTTGGC T-3′; oligo C364S, 5′TCCGCCGATGCTCAATTCGCC-3′; oligo D401N, 5′-CCATTTGGCCAAGTTACCTGTGCGG-3′; oligo D401S, 5′-CCATTTGGCCAAGCTACCTGTGCGG-3′; oligo S562A, 5′-TCGCTTGGATCGCATCTCCGCCGAG C-3′; and oligo S562G, 5′-CGCTTGGATCCCATCTCCG CC-3′. Each mutant plasmid was confirmed by sequencing by the chain termination method of Sanger et al. (52) with the T7 sequencing kit (Pharmacia). Each altered sequence was read in a range of 250 bp around the mutated site. Mutations were observed only in the region where the mutant oligonucleotide hybridized to the template single-stranded DNA.

Expression of TycA. Plasmids containing a wild-type or mutant *tycA* gene were used to transform *E. coli* JM105. Transformants were grown in 2×YT supplemented with ampicillin (50 g/ml) until 12 h after the cells entered the stationary phase. Induction was not necessary because TycA was expressed to high levels in the presence and absence of IPTG (isopropylthiogalactopyranoside). Substantially higher activities in the amino acid-binding assay were obtained from TycA preparations of late-stationary-phase cells than of early-stationary-phase cells.

Enzyme purification. All operations were carried out at 0 to 4°C except the final purification on Mono Q, which was performed at room temperature. Cells were grown in 800 ml of 2×YT medium, harvested in a GS-3 rotor (Sorvall) for 15 min at 6,000 rpm, and resuspended in a minimum volume of buffer A (50 mM Tris [pH 8.0], 2 mM dithioerythritol [DTE], 1 mM EDTA). The suspension was sonified three times on a Branson Sonifier for 1 min each. The cell debris was removed by centrifugation in an SS-34 rotor (Sorvall) for 15 min at 19,000 rpm, and the supernatant was mixed with an equal volume of a saturated ammonium sulfate solution. After being stirred for 1 h, the precipitate was collected by centrifugation in an SS-34 rotor for 15 min at 15,000 rpm. The pellet was dissolved in a minimum volume of buffer A, and 3-ml portions were applied to a Superdex 200 HR 16/60 column. Elution was carried out in buffer A supplemented with 100 mM NaCl. Fractions which showed activity in the phenylalanine-binding assay were pooled, adjusted to 1 M ammonium sulfate, and loaded on a Phenylsepharose HR 16/10 column for hydrophobic interaction chromatography. The column was eluted with a linear descending gradient from buffer A containing 1 M ammonium sulfate to double-distilled water. Active fractions were combined, dialyzed against buffer B (20 mM Tris [pH 8.0], 2 mM DTE, 1 mM EDTA) and applied to an anion-exchange Mono Q HR 5/5 column. Elution was performed with a linear gradient of 0 to 1 M NaCl in buffer B. The highly purified fractions were mixed with sucrose to a final concentration of 10% and stored at −80°C. The mutant enzymes were purified in the same way.

Determination of protein content. After each purification step, the protein concentration of active fractions was measured by the Bradford method (5) with bovine serum albumin as the standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (25). The resolving gel contained 10% acrylamide, 0.1% SDS, 375 mM Tris (pH 8.8), and 0.26% *N,N*-ethylenebisacrylamide. The stacking gel contained 4% acrylamide, 0.1% SDS, 125 mM Tris (pH 6.8), and 0.1% *N,N*-methylenebisacrylamide. The dimensions of gel were 60 by 90 by 0.75 mm. Samples from columns were treated with sample buffer (125 mM Tris [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 5% [vol/vol] mercaptoethanol, 1.25 mg of bromophenol blue per liter) for 15 min at 95°C. Total-cell extracts of *E. coli* were obtained by suspending the cells in TE (10 mM Tris [pH 8.0],

1 mM EDTA) supplemented with lysozyme (1 mg/ml) and incubating them at 37°C for 30 min. The preparation was then mixed with sample buffer and heated (see above). Electrophoresis was performed for 1 h at 40 mA. Gels were stained with Serva Blue R. Gels loaded with β-[^{3}H]Ala-labeled proteins were soaked in Amplify (Amersham), dried, and fluorographed on an X-ray film (Kodak X-OMAT AR).

Assays. ATP-PP_i exchange by TycA was measured essentially as described by Lee and Lipmann (27). Each assay mix contained 10 μg of enzyme per ml, 0.2 mM ATP, 1 mM magnesium acetate, 1 mM DTE, 0.1 mM EDTA, 0.2 mM D-phenylalanine, 0.1 mM Na_4PP_i, and 0.15 Ci of $^{32}PP_i$ in a total volume of 70 μl. This assay was carried out after each step of purification of the wild-type enzyme and after the final purification step of the mutant enzymes.

Covalent binding of amino acid by the peptide synthetase was tested as described previously (59). Each test mix contained 50 μl of enzyme fraction, 50 mM Tris (pH 7.8), 1 mM DTE, 2 mM ATP, 10 mM $MgCl_2$, and 3 μCi of [^{14}C]Phe in a total volume of 100 μl. The mixture was incubated at 37°C for 20 min. The reaction was stopped with 2 ml of ice-cold 7% trichloroacetic acid (TCA), and the mixture was incubated on ice for about 20 min. TCA-precipitated protein was collected on a glass fiber filter (GF92; Schleicher & Schuell, Dassel, Germany) and washed with an excess of TCA, and bound [^{14}C]Phe was counted in a 1900CA Tri-Carb liquid scintillation analyzer (Packard).

Cleavage of thioesterified amino acid was done as described by Ullrich et al. (59). [^{14}C]Phe covalently bound to TycA was precipitated with TCA, and the precipitate was washed five times with TCA and two times with ethanol to remove all traces of unbound phenylalanine and dried in a vacuum. The pellet was resuspended in a small volume of 80% formic acid–6% hydrogen peroxide and incubated at 56°C for 30 min. The reaction mixture was dried under vacuum. The pellet was extracted several times with 50% ethanol, and the combined ethanol phases were subjected to thin-layer chromatography (TLC) for analysis.

TLC. Silica gel SI F plates (20 by 20 cm^2; Riedel-de Haen, Seelze, Germany) were used with solvent system A, 1-butanol–acetic acid–H_2O (40:10:10). Chiral plates (10 by 10 cm^2; Machery-Nagel, Düren, Germany) were used with solvent system B, methanol-H_2O-acetonitrile (50:50:200).

Incorporation of β-[^{3}H]alanine. *E. coli* SJ16 (48) was transformed with pTZ19U*tycA* and maintained on agar plates with minimal medium E (60) supplemented with 0.5% (wt/vol) D-glucose, 0.0001% thiamine, 0.002% methionine, 0.01 mM β-alanine, and 50 μg of ampicillin per ml. A 1-ml overnight culture of these cells was centrifuged, and the pellet was washed four times in medium E without β-alanine to remove all traces of β-alanine. Labeling was done in 2 ml of medium E supplemented with 0.5% (wt/vol) D-glucose, 0.0001% thiamine, 0.002% methionine, 50 μg of ampicillin per ml, and β-[^{3}H]alanine at a final concentration and specific activity of 0.3 μM and 91.5 Ci/mmol, respectively. The medium was inoculated with 2 μl washed cells. Cells grown at 37°C for 24 h in a waterbath shaker were harvested by centrifugation, washed once with TE, and prepared for SDS-PAGE.

A simplified labeling procedure used *E. coli* JM105 with pTZ19U*tycA* in 0.2×YT, which has 1/10 the strength of complete medium 2×YT. A 1-ml amount of 0.2×YT with 50 μg of ampicillin per ml was supplemented with 50 μl of β-[^{3}H]alanine (1 mCi/ml), inoculated with 5 μl of an *E. coli* JM105/pTZ19U*tycA* overnight culture, and incubated for 18 h at 37°C in a waterbath shaker.

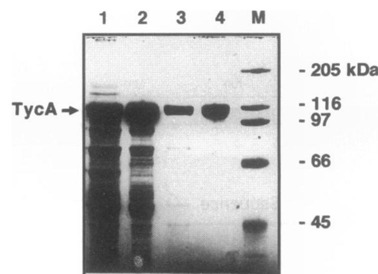

FIG. 2. Purification of TycA. Samples from each purification step were analyzed on an SDS–10% polyacrylamide slab gel (see text). Lane 1, crude extract of *E. coli* JM105/pTZ19U*tycA*; lane 2, fraction after Superdex 200 step; lane 3, enzyme from Phenylsepharose step; lane 4, TycA from Mono Q step; lane M, molecular size markers.

RESULTS

Expression and purification of TycA and TycA mutants from *E. coli*. TycA was overexpressed in *E. coli* JM105/pTZ19U*tycA* without induction by IPTG. Why is not fully understood, since the *tycA* gene in pTZ19U*tycA* does not contain its own promoter (36). Within the region just upstream of *tycA*, however, are two sets of sequences that could potentially serve as promoter sites for the major form of *E. coli* RNA polymerase (unpublished data).

After disintegration of cells, only half of the TycA preparation was soluble, while the other half remained in the insoluble fraction. This was reported in an earlier study of TycA, in which localization to the cytoplasmic membrane was suggested (23). The soluble TycA was purified by 50% ammonium sulfate precipitation, gel filtration on Superdex 200 HR 16/60, hydrophobic interaction chromatography on Phenylsepharose HR 16/10, and anion-exchange chromatography on Mono Q HR 5/5 as described in Materials and Methods. The result of each purification step is shown in Fig. 2. The enzyme specific activity was measured after each step by D-phenylalanine-dependent ATP-PP_i exchange (Table 1). After purification, homogeneous TycA showed a 6.2-fold increase in specific activity.

Single-amino-acid changes in TycA (Fig. 3) were introduced by site-directed mutagenesis of *tycA* (57). New amino acids were selected for "safe" residue substitutions (4) to minimize the risk of conformational changes in TycA. In core 2, we replaced each of three glycines with alanine, the proline residue with valine, and the second lysine (residue 186) with arginine or threonine. Cysteine 364 of core 3 was replaced by serine. In core 4, aspartate 401 was replaced by asparagine or serine. During analysis of the mutants, an unexpected triple mutation was observed. Instead of two changed nucleotides leading to an Asp-401-to-Ser mutation, two additional nucle-

TABLE 1. Purification of TycA

Step	Total protein (mg)	Total activity[a] (nkat)	Sp act (nkat/mg)	Recovery (%)	Purification (fold)
Crude extract	548	805	1.47	100	1
Ammonium sulfate fractionation	153	643	4.20	80	2.9
Superdex 200	106	614	5.78	76	3.9
Phenylsepharose	35.6	273	7.58	34	5.2
Mono Q	21.8	197	9.07	24	6.2

[a] One nanokatal (nkat) is the amount of enzyme catalyzing the incorporation of 1 nmol of PP_i into ATP per second.

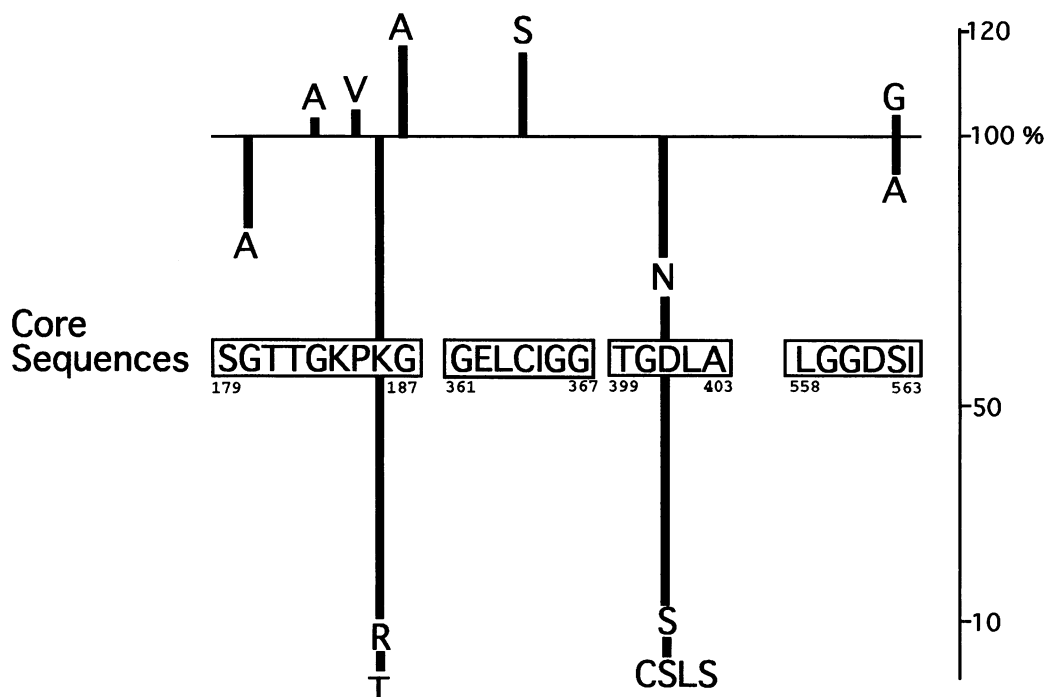

FIG. 3. Influence of point mutations in TycA on D-phenylalanine-dependent ATP-PP_i exchange activity. The sequences of cores 2, 3, 4, and 6, with the corresponding numbering of amino acids in TycA, are shown in the center. Vertical bars represent the deviation in ATP-PP_i exchange activity for each mutation relative to the wild-type level. The right-hand scale represents the activity values.

otides were replaced, leading to a change of three amino acids. Serine 562 of core 6 was replaced by glycine or alanine.

Expression of the mutant proteins was comparable to wild-type expression except for core 4 mutants. The Asp-401-to-Ser mutant was expressed only to about 1/10 of the level of the wild type, as judged by SDS-PAGE analysis. Other mutations of glycine 400 in core 4 resulted in expression, but the proteins were retained completely in the insoluble fraction.

ATP-PP_i exchange. Figure 3 shows the influence of mutations in cores 2, 3, 4, and 6 on phenylalanine-dependent ATP-PP_i exchange activity. Variations of the three glycines in core 2 caused no significant alteration in exchange activity compared with the wild type. The proline-to-valine mutant also showed the activity level of the wild type. Only the two lysine-186 mutations caused significant loss of activity. While the replacement of lysine with the basic amino acid arginine caused a 90% reduction in activity, the replacement of lysine with the neutral threonine reduced the activity to less than 1% (Table 2). Mutations in core 4 also decreased exchange activity, but to a smaller degree than did mutations in core 2. TycA with an aspartic acid-to-asparagine mutation showed a decrease of 78% in exchange activity, while the aspartic acid 401-to-serine mutation caused only a 12% reduction in activity. The exchange activity of the triple mutant was reduced to 3% of the wild-type level. Replacement of cysteine 364 with serine in core 3 or of the serine in core 6 with glycine or alanine had no significant influence on ATP-PP_i exchange activity compared with the wild-type level.

Covalent binding of phenylalanine to TycA and cleavage of thioesterified TycA-phenylalanine complex. Covalent binding of phenylalanine to recombinant TycA from *E. coli* is very inefficient. Only 1 to 1.5% of TycA from *E. coli* can be charged with phenylalanine, compared with 40% for TycA from *B. brevis*. Figure 4A shows a comparison of the phenylalanine-binding capacity of TycA and three mutants with alterations in cores 3 and 6. The cysteine-to-serine core 3 mutation led to an increased binding capacity of about 60% over the wild-type level, whereas the serine-to-alanine and serine-to-glycine mutations of core 6 showed a decrease in binding capacity down to 30% of the wild-type level. These complexes were stable to acid but decayed after treatment with performic acid. Only the wild-type and the cysteine-to-serine mutant showed free phenylalanine after performic cleavage, whereas the serine-to-

TABLE 2. D-Phenylalanine-dependent ATP-PP_i exchange activities of TycA mutants

Core	Mutation[a]	Exchange activity (cpm/min/μg)	% of wild-type activity
—	None (wild type)	9,499	100
2	G180A	7,789	82
	G183A	9,689	102
	P185V	9,784	103
	K186R	950	10
	K186T	47	0.5
	G187A	11,114	117
3	C364S	10,924	115
4	D401N	7,409	78
	D401S	1,139	12
	G400C, D401S, A403S	285	3
6	S562G	9,772	103
	S562A	8,834	93

[a] Mutations shown as original residue and position followed by new mutant residue.

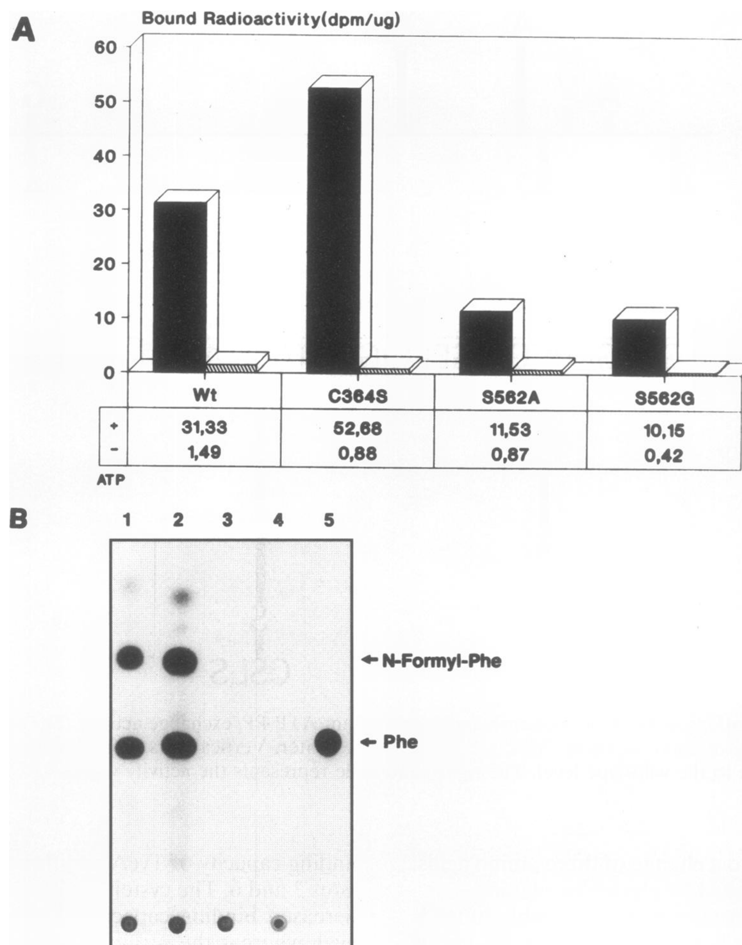

FIG. 4. (A) Charging of wild-type and mutant TycA with [^{14}C]phenylalanine. Wild-type (WT) TycA and three mutants, C364S (cysteine 364 to serine), S562A (serine 562 to alanine), and S562G (serine 562 to glycine), were charged with [^{14}C]phenylalanine in the presence (+) and absence (−) of ATP. The vertical bars represent the radioactivity (disintegrations per minute) of TCA-precipitable TycA-[^{14}C]phenylalanine complex per microgram of enzyme. (B) Cleavage of TycA- and mutant TycA-[^{14}C]phenylalanine complexes with performic acid and separation of cleavage products by silica gel TLC. Lane 1, TycA; lane 2, C364S; lane 3, S562A; lane 4, S562G; lane 5, [^{14}C]phenylalanine. A part of phenylalanine is converted to *N*-formyl-phenylalanine after treatment with performic acid (34).

alanine and serine-to-glycine mutants showed no cleavable phenylalanine after separation by silica gel TLC and subsequent autoradiography (Fig. 4B).

Racemization of phenylalanine. To determine the racemization capacity of mutant TycA, we introduced a simple new test. After incubation of TycA with L-[^{14}C]Phe, the generated thioester was cleaved with performic acid. The separated [^{14}C]phenylalanine was then subjected to chiral plate TLC and autoradiographed. The retention values were compared with published data. The L- and D-forms of phenylalanine were easily distinguished by their R_f values of 0.59 and 0.49, respectively. Wild-type TycA and the cysteine 364-to-serine core 3 mutant displayed no difference in phenylalanine racemization, whereas the serine 562-to-alanine and serine 562-to-glycine core 6 mutants produced no detectable signal (Fig. 5).

Incorporation of β-[^{3}H]alanine. Although core 6 of TycA shows strong similarity to a pantetheine-binding site (54), pantetheine was found only in TycB (29) and TycC (23) but not in TycA in a microbiological pantothenic acid assay (43). We used the in vivo incorporation of ^{3}H-labeled β-alanine to check the 4′-phosphopantetheine content of TycA. pTZ19U*tycA* was used to transform *E. coli* SJ16, which is a *panD* mutant that is

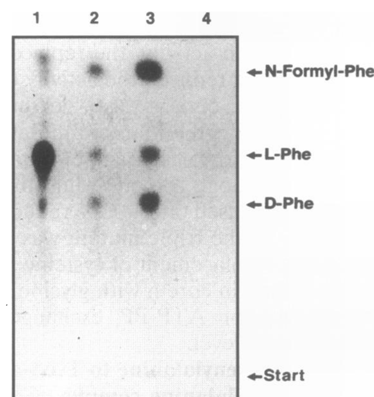

FIG. 5. Analysis of TycA-[^{14}C]phenylalanine cleavage products by chiral plate TLC. Lane 1, control; commercially available L-[^{14}C]Phe slightly contaminated with D-[^{14}C]Phe; lane 2, TycA; lane 3, C364S; lane 4, S562A.

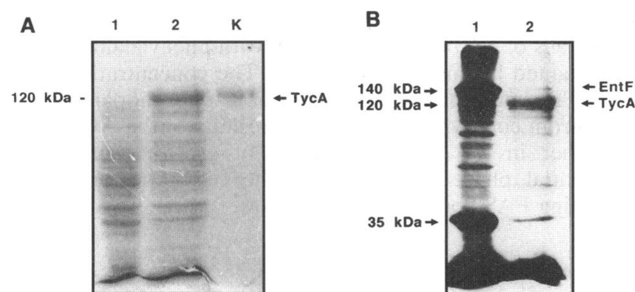

FIG. 6. Analysis of β-[^{3}H]alanine labeling of *E. coli* JM105. (A) SDS–10% PAGE of labeled *E. coli* extracts. Lane 1, crude extract of *E. coli* SJ16/pTZ19U*tycA* grown overnight in minimal medium E supplemented with 0.5% (wt/vol) D-glucose, 0.0001% thiamine, 0.002% methionine, 50 g of ampicillin per ml, and β-[^{3}H]alanine at a final concentration and specific activity of 0.5 M and 80 Ci/mmol, respectively; lane 2, crude extract of *E. coli* JM105/pTZ19U*tycA* grown overnight in 0.2×YT supplemented with ampicillin (50 μg/ml) and β-[^{3}H]alanine at a final concentration of 0.5 μM; lane K, TycA. (B) Fluorography of SDS-PAGE gel from panel A. Lane 1, crude extract of *E. coli* SJ16/pTZ19U*tycA*; lane 2, crude extract of *E. coli* JM105/pTZ19U*tycA*.

defective in the biosynthesis of β-alanine, the precursor of 4′-phosphopantetheine (19). Cells were starved for β-alanine by growth on minimal medium E plates and inoculated in liquid medium E supplemented with β-[^{3}H]alanine. After 24 h, the cells were harvested, and extracts were analyzed by SDS-PAGE and fluorography. Several proteins with apparent molecular masses of about 20, 35, and 140 kDa were found to be labeled. While the 20-kDa protein represents free acyl carrier protein (ACP) (3, 8, 20), the 35-kDa protein represents an unknown protein whose existence has been reported previously (45, 47). The 140-kDa labeled protein represents EntF, a protein involved in enterobactin biosynthesis (50). This protein is induced by iron starvation and was suppressed in minimal medium supplemented with 10 μM $FeSO_4$ (data not shown). No labeling of TycA could be detected under these conditions in *E. coli* SJ16.

Since TycA was very weakly expressed in *E. coli* SJ16/pTZ19U*tycA* grown in minimal medium E, we modified the growth conditions to improve the expression of TycA, accepting a lower β-alanine incorporation efficiency. *E. coli* JM105 was transformed with pTZ19U*tycA* and labeled with β-[^{3}H]alanine for 18 h in 0.2×YT medium. Cell extracts were then analyzed for the incorporation of β-[^{3}H]alanine by SDS-PAGE and fluorography. The diluted rich medium (0.2×YT) ensures strong expression of TycA and also allows good incorporation of β-[^{3}H]alanine. As shown in Fig. 6A, the expression of TycA is much stronger in *E. coli* JM105/pTZ19U*tycA* grown in 0.2×YT (lane 2) than in *E. coli* SJ16/pTZ19U*tycA* grown in medium E (lane 1). The fluorograph in Fig. 6B shows predominantly EntF and the 35-kDa protein in the overexposure of lane 1 (*E. coli* SJ16/pTZ19U*tycA*, medium E) and a labeled TycA in lane 2 (*E. coli* JM105/pTZ19U*tycA*, 0.2×YT). In addition to TycA, weak signals for EntF and the 35-kDa protein were visible. The intensity of the 35-kDa protein in Fig. 6A, lanes 1 and 2, indicates the difference in labeling efficiency in the two experiments and emphasizes the specific labeling of TycA in lane 2. This specific incorporation of β-alanine in TycA is a strong indication for the modification of this multienzyme with 4′-phosphopantetheine. In a control experiment carried out in 0.2×YT medium with *E. coli* JM105 bearing pTZ19U which did not contain the *tycA* gene, no signal of the size of TycA could be detected (not shown).

DISCUSSION

Twelve TycA mutants were examined for their D-phenylalanine-dependent ATP-PP_i exchange activity, for their racemization capacity, and for their capacity to bind phenylalanine as a thioester. The incorporation of β-alanine into TycA was also studied in vivo to determine the 4′-phosphopantetheine content in TycA.

Only mutations which altered lysine 186 in core 2 or aspartate 401 in core 4 caused a significant decrease in specific ATP-PP_i exchange activity. Although core 2 (SGTTGKPKG) shows only poor homology to the Walker A type motif, GXXXXGK(T/S) (16, 63), it resembles this motif in that it is rich in glycine and contains a lysine. This motif forms a so-called phosphate-binding loop, which is found in all guanosine and some adenosine nucleotide-binding proteins (53). The lysine is supposed to bind the γ-phosphate of the nucleoside triphosphate. The ATP-binding site of the plasma membrane H^+-ATPase of *Neurospora crassa* was determined to be lysine by labeling with fluorescein 5′-isothiocyanate, a derivative of ATP (40). Lysine was also identified as the ATP-binding site of SecA, a protein involved in protein transport across the *E. coli* plasma membrane (24). Therefore, replacement of lysine with another basic amino acid should cause a decrease to a residual TycA enzyme activity, whereas replacement with an amino acid with altered functionality should cause loss of activity. This was confirmed by the results with the lysine-to-arginine and lysine-to-threonine mutants of TycA.

The TGD motif of core 4 is a widespread motif among cation-ATPases. The K^+-ATPase of *E. coli*, the (Na^++K^+)-ATPase from sheep kidney, the Ca^{2+}-ATPase from rabbit muscle (55), and H^+-ATPase from *N. crassa* (1) show identity in this motif. Labeling of yeast plasma membrane H^+-ATPase with 2-azido-ATP revealed a nine-residue peptide containing the TGD motif (9). Furthermore, a mutation of aspartate to asparagine dramatically decreased the specific activity of this ATPase (41). Similar results are presented for TycA. Changing aspartate to asparagine decreased the ATP-PP_i exchange activity to 78% of the wild-type level, whereas an exchange to serine decreased the activity to 12%. The triple mutation probably changes the conformation of the active site and is therefore responsible for loss of activity. Why the aspartate-to-asparagine mutant is more active than the aspartate-to-serine mutant is not clear. These two series of experiments confirm the role of lysine 186 in core 2 and aspartate 401 in core 4 as essential residues in the interaction of TycA with ATP. Since a crystallographic study of a TycA-ATP complex is not yet available, direct evidence for the ATP-binding site or the site of ATPase activity is lacking.

To study the formation of the TycA phenylalanine thioester linkage, TycA mutants with alterations in core 3 and core 6 were tested for phenylalanine incorporation. Core 3 contains the only cysteine which is conserved in a limited number of peptide synthetases and thus represents the sole candidate for direct thioester formation without an adapter molecule. Since the cysteine 364-to-serine mutant shows an even higher binding capacity for phenylalanine than the wild type and is cleaved by performic acid in the same way as the wild type, the possibility of direct thioester formation can be excluded. Alteration of serine 562 in the putative 4′-phosphopantetheine-binding site to alanine or glycine causes a reduction in the residual precipitable TycA-Phe complex level to one-third of

the wild-type level, but no phenylalanine is removed after treatment with performic acid. Since no labeled phenylalanine was gained after treatment with performic acid, we conclude that no cleavable thioester was present.

It seems that, to some extent, the precursor of the thioester, the phenylalanyladenylate, complexes with TycA and is coprecipitated by TCA treatment. Why this complex remains stable in strong acid such as TCA is still unclear. The absence of any signal in the autoradiograph of TLC preparations could be explained by the loss of the labeled compound, which shows altered solubility during the extraction procedure. This suggests that serine 562 of core 6 is the site for thioester formation in TycA. No loss of D-Phe-dependent ATP-PP_i exchange activity was detected in the serine 562-to-alanine or -glycine mutants, which is in contrast to an observation of Vollenbroich et al. (61). They reported the loss of glutamate-dependent ATP-PP_i exchange activity for a serine-to-alanine mutation in the corresponding core of the first domain of surfactin synthetase E_{1A}. This enzyme catalyzes the incorporation of glutamate, leucine, and D-leucine into the lipopeptide surfactin. The reason for this discrepancy is not clear.

Earlier studies on GrsA (gramicidin S synthetase 1) used enantiomer-specific amino acid oxidase for determination of racemase activity (65). Separation of the cleavage products by chiral TLC represents a new alternative for monitoring racemase activity. The TycA mutant with a change of cysteine to serine in core 4 (GELCIGG) retained full wild-type racemization capacity (Fig. 5). A comparison of the amino acid-racemizing domains of peptide synthetases revealed four homologous motifs at the C-terminal end of each domain (12), which were not the subject of this investigation. The racemization reaction of GrsA is supposed to occur on the thioesterified phenylalanine, and a base group has been suggested to function as a proton donor and acceptor during racemization (21). Site-directed mutagenesis of the mentioned racemase motifs of TycA and subsequent analysis by chiral plate TLC should identify the amino acid responsible for racemization. This work is in progress in our group.

To determine the 4′-phosphopantetheine content, TycA was labeled in vivo with β-[^{3}H]alanine. Labeling was successful in *E. coli* JM105 in 0.2× YT, but no clear signals were visible in *E. coli* SJ16 in medium E. For labeling in *E. coli*, the correct action of holo-ACP synthase is required. Holo-ACP normally catalyzes the transfer of 4′-phosphopantetheine from coenzyme A to ACP. NodF, a protein of *Rhizobium leguminosarum* which is involved in the nodule-forming process in plants, is labeled with β-alanine in *E. coli*, indicating that it is recognized by the ACP synthase of *E. coli* (14). Several ACPs from heterologous sources are correctly modified in *E. coli*. The ACP of *Saccharopolyspora erythraea* was shown to be expressed in *E. coli* and modified with 4′-phosphopantetheine to functionality (44). Spinach ACP (15) and the ACP of tetracenomycin C polyketide synthase TcmM of *Streptomyces glaucescens* (56) were modified with 4′-phosphopantetheine in *E. coli*, as demonstrated by incorporation of β-[^{3}H]alanine.

Our special interest is the incorporation of β-[^{3}H]alanine into peptide synthetases. EntF activates L-serine in the biosynthesis of enterobactin in *E. coli* and is labeled in vivo with β-alanine (50), whereas 6-deoxyerythronolide B synthase 3 of *S. erythraea*, which is involved in the synthesis of the polyketide erythromycin A, could not be labeled in *E. coli* even though it shows the 4′-phosphopantetheine-binding motif of ACPs (45). We suggest that TycA is a poor substrate for the ACP synthase of *E. coli* and that therefore modification of TycA with 4′-phosphopantetheine is incomplete. This explains the low level (only 1.5%) of TycA which can be charged with phenylalanine. The observation that only TycA from late-stationary-phase cells of *E. coli* can be charged with phenylalanine could be explained by another hypothesis. The concentration of *E. coli* ACP synthase that transfers the 4′-phosphopantetheine group from coenzyme A to a serine residue of the ACP could be higher in the stationary phase of growth than in the exponential phase of growth, or the concentration of the competing FAS apo-ACP could be lower (42).

ACKNOWLEDGMENTS

Anti-TycA antibodies were a gift from E. Pfeifer (Institut für Biochemie und Molekulare Biologie, Universität Berlin, Berlin, Germany), and *E. coli* SJ16 was a gift from C. O. Rock (Dept. of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tenn.). We thank E. Pfeifer and M. Pavela-Vrancic (Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, Berlin, Germany) for improvements of ATP-PP_i exchange reactions. We also thank S. Borchert, N. Rajendran, K. Turgay, and P. Zuber for comments on the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft, the Graduiertenkolleg "Enzymchemie," and in part by the Fond der Chemischen Industrie.

REFERENCES

1. **Addison, R.** 1986. Primary structure of the *Neurospora* plasma membrane H^+-ATPase deduced from the gene sequence. J. Biol. Chem. **261:**14896–14901.
2. **Akers, H. A., S. G. Lee, and F. Lipmann.** 1977. Identification of two enzymes responsible for the synthesis of the initial portion of linear gramicidin. Biochemistry **16:**5722–5729.
3. **Alberts, A. W., and P. R. Vagelos.** 1966. Acyl carrier protein. J. Biol. Chem. **241:**5201–5204.
4. **Bordo, D., and P. Argos.** 1991. Suggestions for "safe" residue substitutions in site-directed mutagenesis. J. Mol. Biol. **217:**721–729.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities using the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, T. A.** 1991. Molecular biology labfax. BIOS Scientific Publishers Limited, Manchester, United Kingdom.
7. **Cosmina, P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen.** 1993. Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Mol. Microbiol. **8:**821–831.
8. **Cronan, J. E.** 1980. β-Alanine synthesis in *Escherichia coli*. J. Bacteriol. **141:**1291–1297.
9. **Davis, C. B., K. E. Smith, B. N. Campbell, Jr., and G. G. Hammes.** 1990. The ATP binding site of the yeast plasma membrane proton-translocating ATPase. J. Biol. Chem. **265:**1300–1305.
10. **De Wet, J. R., K. V. Wood, M. De Luca, D. R. Helsinki, and S. Subrami.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
11. **D'Souza, C., M. M. Nakano, N. Corbell, and P. Zuber.** 1993. Amino-acylation site mutations in amino acid-activating domains of surfactin synthetase: effects on surfactin production and competence development in *Bacillus subtilis*. J. Bacteriol. **175:**3502–3510.
12. **Fuma, S., Y. Fujishima, N. Corbell, C. D'Souza, M. M. Nakano, P. Zuber, and K. Yamane.** 1993. Nucleotide sequence of 5′ portion of *srfA* that contains the region required for competence establishment in *Bacillus subtilis*. Nucleic Acids Res. **21:**93–97.
13. **Fürbass, R., M. Gocht, P. Zuber, and M. A. Marahiel.** 1991. Interaction of AbrB, a transcriptional regulator from *Bacillus subtilis*, with the promoters of the transition state-activated genes *tycA* and *spoVG*. Mol. Gen. Genet. **225:**347–354.
14. **Geiger, O., H. P. Spaink, and E. P. Kennedy.** 1991. Isolation of the *Rhizobium leguminosarum* NodF nodulation protein: NodF carries a 4′-phosphopantetheine prosthetic group. J. Bacteriol. **173:**2872–2878.
15. **Guerra, D. J., K. Dziewanoska, J. B. Ohlrogge, and P. D. Beremand.** 1988. Purification and characterization of recombinant

spinach acyl carrier protein I expressed in *Escherichia coli*. J. Biol. Chem. **263**:4386–4391.
16. **Higgins, C. F., I. D. Hiles, G. P. C. Salmond, D. R. Gill, J. A. Downie, I. J. Evans, I. B. Holland, L. Gray, S. D. Buckel, A. W. Bell, and M. A. Hermodson.** 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature (London) **323**:448–450.
17. **Hori, K., Y. Yamamoto, T. Minetoki, T. Kurotsu, M. Kanda, S. Miura, K. Okamura, J. Furujama, and J. Saito.** 1989. Molecular cloning and nucleotide sequence of the gramicidin S synthetase 1 gene. J. Biochem. **106**:639–645.
18. **Hotchkiss, R. D., and R. J. Dubos.** 1940. Fractionation of the bacterial agent from cultures of a soil bacillus. J. Biol. Chem. **132**:791–792.
19. **Jackowski, S., and C. O. Rock.** 1981. Regulation of coenzyme A biosynthesis. J. Bacteriol. **148**:926–932.
20. **Jackowski, S., and C. O. Rock.** 1983. Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **258**:15186–15191.
21. **Kanda, M., K. Hori, T. Kurotsu, S. Miura, and Y. Saito.** 1989. Reaction mechanism of gramicidin S synthetase 1, phenylalanine racemase, of *Bacillus brevis*. J. Biochem. **105**:653–659.
22. **Kleinkauf, H., W. Gevers, R. Roskoski, Jr., and F. Lipmann.** 1970. Enzyme-bound phosphopantetheine in tyrocidine biosynthesis. Biochem. Biophys. Res. Commun. **41**:1218–1222.
23. **Kleinkauf, H., and H. von Döhren.** 1990. Nonribosomal biosynthesis of peptide antibiotics. Eur. J. Biochem. **192**:1–15.
24. **Klose, M., K.-L. Schimz, J. van der Wolk, A. J. M. Driessen, and R. Freudl.** 1993. Lysine 106 of the putative catalytic ATP-binding site of the *Bacillus subtilis* SecA protein is required for functional complementation of *Escherichia coli secA* mutants *in vivo*. J. Biol. Chem. **268**:4505–4510.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lee, S. G., and F. Lipmann.** 1974. Isolation of a peptidyl-pantetheine-protein from tyrocidine-synthesizing polyenzymes. Proc. Natl. Acad. Sci. USA **71**:607–611.
27. **Lee, S. G., and F. Lipmann.** 1975. Tyrocidine synthetase system. Methods Enzymol. **43**:585–602.
28. **Lee, S. G., and F. Lipmann.** 1977. Isolation of amino acid activating subunit-pantetheine protein complex. Proc. Natl. Acad. Sci. USA **74**:2343–2347.
29. **Lee, S. G., R. Roskoski, K. Bauer, and F. Lipmann.** 1973. Purification of the polyenzymes responsible for tyrocidine synthesis and their dissociation into subunits. Biochemistry **12**:398–405.
30. **Lipmann, F.** 1980. Bacterial production of antibiotic polypeptides by thiol-linked synthesis on protein templates. Adv. Microb. Physiol. **21**:227–266.
31. **Lipmann, F., W. Gevers, H. Kleinkauf, and R. Roskoski.** 1971. Polypeptide synthesis on proteine templates: the enzymatic synthesis of gramicidinS and tyrocidines. Adv. Enzymol. **35**:1–33.
32. **Loyoza, E., H. Hoffmann, C. Douglas, W. Schulz, D. Scheel, and K. Hahlbrock.** 1988. Primary structure and catalytic properties of isoenzymes encoded by the two 4.coumarate:CoA ligase genes in parsley. Eur. J. Biochem. **176**:661–667.
33. **Marahiel, M. A.** 1992. Multidomain enzymes involved in peptide synthesis. FEBS **307**:40–43.
34. **Marahiel, M. A., M. Krause, and H.-J. Skarpeid.** 1985. Cloning of the tyrocidine synthetase 1 gene from *Bacillus brevis* and its expression in *Escherichia coli*. Mol. Gen. Genet. **201**:231–236.
35. **Marahiel, M. A., M. M. Nakano, and P. Zuber.** 1993. Regulation of peptide antibiotic production in *Bacillus*. Mol. Microbiol. **7**:631–636.
36. **Marahiel, M. A., P. Zuber, G. Czekay, and R. Losick.** 1987. Identification of the promoter for a peptide antibiotic biosynthesis gene from *Bacillus brevis* and its regulation in *Bacillus subtilis*. J. Bacteriol. **169**:2215–2222.
37. **Marinus, M. G., and N. R. Morris.** 1973. Isolation of deoxyribonucleic acid methylase mutants of *Escherichia coli* K-12. J. Bacteriol. **114**:1143–1148.
38. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
39. **Mittenhuber, G., R. Weckermann, and M. A. Marahiel.** 1989. Gene cluster containing the genes for tyrocidine synthetases 1 and 2 from *Bacillus brevis*: evidence for an operon. J. Bacteriol. **171**:4881–4887.
40. **Pardo, J. P., and C. W. Slayman.** 1988. The fluorescein isothiocyanate-binding site of the plasma-membrane H^+-ATPase of *Neurospora crassa*. J. Biol. Chem. **263**:18664–18668.
41. **Portillo, F., and R. Serrano.** 1988. Dissection of functional domains of the yeast proton-pumping ATPase by directed mutagenesis. EMBO J. **7**:1793–1798.
42. **Prescott, D. J., and P. R. Vagelos.** 1972. Acyl carrier protein. Adv. Enzymol. **36**:269–311.
43. **Pugh, E. L., and S. G. Wakil.** 1965. Studies on the mechanism of fatty acid synthesis. J. Biol. Chem. **240**:4727–4732.
44. **Revill, W. P., and P. Leadlay.** 1991. Cloning, characterization, and high-level expression in *Escherichia coli* of the *Saccharopolyspora erythraea* gene encoding an acyl carrier protein potentially involved in fatty acid biosynthesis. J. Bacteriol. **173**:4379–4385.
45. **Roberts, G. A., J. Staunton, and P. Leadlay.** 1993. Heterologous expression in *Escherichia coli* of an intact multienzyme component of the erythromycin-producing polyketide synthase. Eur. J. Biochem. **214**:305–311.
46. **Robertson, J. R., M. Gocht, M. A. Marahiel, and P. Zuber.** 1989. AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. Proc. Natl. Acad. Sci. USA **86**:8457–8461.
47. **Rock, C. O.** 1982. Mixed disulfides of acyl carrier protein and coenzyme A with specific soluble proteins in *Escherichia coli*. J. Bacteriol. **152**:1298–1300.
48. **Rock, C. O., and J. Cronan, Jr.** 1981. Acyl carrier protein from *Escherichia coli*. Methods Enzymol. **71**:341–351.
49. **Roskoski, R., Jr., H. Kleinkauf, W. Gevers, and F. Lipmann.** 1970. Isolation of enzyme-bound peptide intermediates in tyrocidine biosynthesis. Biochemistry **9**:4846–4849.
50. **Rusnak, F., M. Sakaitani, D. Drueckhammer, J. Reichert, and C. T. Walsh.** 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry **30**:2916–2927.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
53. **Saraste, M., P. R. Sibbald, and A. Wittighofer.** 1990. The P-loop: a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15**:430–434.
54. **Schlumbohm, W., T. Stein, C. Ullrich, J. Vater, M. Krause, M. A. Marahiel, V. Kruft, and B. Wittmann-Liebold.** 1991. An active serine is involved in covalent substrate amino acid binding at each reaction center of gramicidin S synthetase. J. Biol. Chem. **266**: 23135–23141.
55. **Serrano, R., M. C. Kielland-Brandt, and G. R. Fink.** 1986. Yeast plasma membrane ATPase is essential for growth and has homology with $(Na^+ + K^+)$, K^+- and Ca^{2+}-ATPases. Nature (London) **319**:689–693.
56. **Shen, B., R. G. Summers, H. Gramajo, M. J. Bibb, and C. R. Hutchinson.** 1992. Purification and characterization of the acyl carrier protein of the *Streptomyces glaucescens* tetracenomycin C polyketide synthase. J. Bacteriol. **174**:3818–3821.
57. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13**:8764–8785.
58. **Turgay, K., M. Krause, and M. A. Marahiel.** 1992. Four homologous domains in the primary structure of GrsB are related to domains in a superfamily of adenylate-forming enzymes. Mol. Microbiol. **6**:529–546.
59. **Ullrich, C., B. Kluge, Z. Palacz, and J. Vater.** 1991. Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*. Biochemistry **30**:6503–6508.

60. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
61. **Vollenbroich, D., B. Kluge, C. D'Souza, P. Zuber, and J. Vater.** 1993. Analysis of a mutant amino acid-activating domain of surfactin synthetase bearing a serine-to-alanine substitution at the site of carboxylthioester formation. FEBS Lett. **325**:220–224.
62. **Vollenbroich, D., N. Mehta, P. Zuber, J. Vater, and R. M. Kamp.** 1994. Analysis of surfactin synthetase subunits in *srfA* mutants of *Bacillus subtilis* OKB105. J. Bacteriol. **176**:395–400.
63. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**:945–951.
64. **Weckermann, R., R. Fürbass, and M. A. Marahiel.** 1988. Complete nucleotide sequence of the *tycA* gene coding for the tyrocidine synthetase 1 from *Bacillus brevis*. Nucleic Acids Res. **16**:11841.
65. **Yamada, M., and K. Kurahashi.** 1968. Adenosine triphosphate and pyrophosphate dependent phenylalanine racemase of *Bacillus brevis* Nagano. J. Biol. Chem. **63**:59–69.