Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases

Torsten Stachelhaus, Anja Hüser and Mohamed A Marahiel

Background: A structurally diverse group of bioactive peptides is synthesized by peptide synthetases which act as templates for a growing peptide chain, attached to the enzyme via a thioester bond. The protein templates are composed of distinctive substrate-activating modules, whose order dictates the primary structure of the corresponding peptide product. Each module contains defined domains that catalyze adenylation, thioester and peptide bond formation, as well as substrate modifications. To show that a putative thiolation domain (PCP) is involved in covalent binding and transfer of amino acyl residues during non-ribosomal peptide synthesis, we have cloned and biochemically characterized that region of tyrocidine synthetase 1, TycA.

Results: The 327-bp gene fragment encoding PCP was cloned using its homology to the genes for the acyl carrier proteins of fatty acid and polyketide biosynthesis. The protein was expressed as a His_6 fusion protein, and purified in a single step by affinity chromatography. Incorporation of β-[^{3}H]alanine, a precursor of coenzyme A, demonstrated the modification of PCP with the cofactor 4′-phosphopantetheine. When an adenylation domain is present to supply the amino adenylate moiety, PCP can be acylated *in vitro*.

Conclusions: PCP can bind covalently to the cofactor phosphopantetheine and can subsequently be acylated, strongly supporting the multiple carrier model of non-ribosomal peptide synthesis. The adenylation and thiolation domains can each act as independent multifunctional enzymes, further confirming the modular structure of peptide synthases, and can also perform sequential steps in *trans*, as do multienzyme complexes.

Address: Biochemie-Fachbereich Chemie, Philipps-University of Marburg, Hans-Meerwein-Str., D-35032 Marburg, Germany.

Correspondence: Mohamed A Marahiel
E-mail: marahiel@ps1515.chemie.uni-marburg.de

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Introduction

Biologically active, low molecular weight peptides, lipopeptides, depsipeptides and peptidolactones are found in nearly all groups of microbial organisms. Because of their remarkable structural and functional diversity, these secondary metabolites have attracted great interest in medicine, agriculture and biological research. These linear, cyclic and branched peptide chains include non-proteinogenic, hydroxy- and D-amino acids and may also be modified further through *N*-methylation, acylation, glycosylation or heterocyclic ring formation. About 300 different residues are known to be incorporated into these peptide secondary metabolites [1–3]. The ribosomal machinery, which is mainly restricted to linear chains and a limited set of 21 proteinogenic amino acids, clearly cannot achieve this enormous structural diversity alone. A distinct system, which uses a protein template, is required to introduce the wide range of variability into peptide synthesis [1–5].

The mechanism of substrate activation in this 'non-ribosomal' peptide synthesis depends on a two-step reaction that requires amino acyladenylation and subsequent thioesterification on a distinctive thiol group [1–5]. As in fatty acid synthesis, peripheral cysteine residues were initially proposed as thiotemplate sites and a single intrinsic 4′-phosphopantetheine (4′-PP) cofactor was assumed to be the carrier. This cofactor was thought to interact consecutively with the thioesterified amino acids, guiding the stepwise elongation reaction of the growing peptide chain. More recently, analysis of various protein templates (peptide synthetases) has revealed that they are composed of a series of modular activating units, each equipped with its own 4′-PP prosthetic group which is required for covalent binding to the substrate [6–8]. According to this model, each activated amino acid is presented as a thioester on a protein template via a unique 4′-PP group. This mode of action has been designated the multiple-carrier thiotemplate mechanism [5,9].

In this template-driven mechanism, the multifunctional peptide synthetase would be expected to possess a highly conserved and ordered structure of (semi-)autonomous modules. Previous protein chemical studies revealed proteolytic fragments of ~120 kDa, in which each fragment can activate an individual amino acid [10–14]. Our understanding of the relationship between structure and function in these enzymes has been enhanced by the elucidation of the primary structures of an increasing number of peptide synthetases [15–20]. Sequence comparisons revealed that

a number of conserved modules, each ~1000–1400 amino acids long, are arranged in different patterns in these enzymes; it is the identity of these modules, and the order in which they occur, that deter-mines the sequence of the corresponding peptide product [2,4,5,17,21]. The repeated modules are themselves composed of a linear arrangement of conserved domains representing the enzyme activities involved in the catalysis of substrate activation, modification and condensation [5,21–23]. Recently, dissection studies clearly indicated the relative locations and sequence limits of these domains (Fig. 1) that are involved in substrate recognition and ATP-dependent adenylation (550 amino acids), thioester formation (~100 amino acids), condensation (400 amino acids), and in some cases epimerization (400 amino acids) or *N*-methylation (400 amino acids) [5,22].

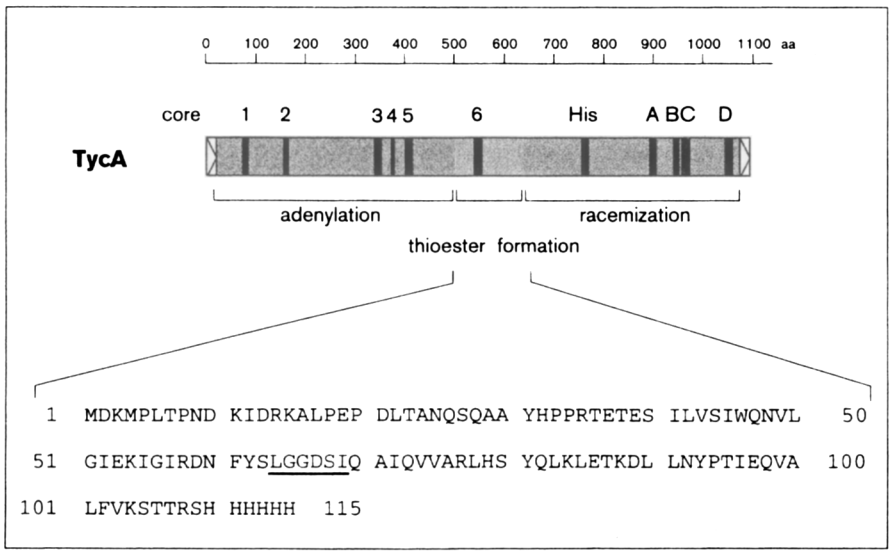
The ordered domains of each amino-acid-activating module are assembled from a linear array of highly conserved core sequences (Fig. 1; [2,4,17]). Many studies, including site-directed mutagenesis and photoaffinity labeling, allowed the specific functions of non-ribosomal peptide biosynthesis to be assigned to individual core motifs. For example, these investigations suggested the involvement of core motifs 2–5 in ATP binding and ATP hydrolysis [8,24–27]. Also, recent studies have provided strong evidence that the 4′-PP prosthetic group is attached to the conserved serine residue of core motif 6, and that the cysteamine thiol group of the cofactor represents the thioester binding site for the amino acid substrate [6–9].

The modular arrangement of the peptide synthases and their strategy of product assembly is extremely reminiscent of the type I fatty acid synthases (FASs) and polyketide synthases (PKSs), with which they also share sequence homology [28–30]. FASs and PKSs are multifunctional enzymes that catalyze a repeated decarboxylative condensation, commonly between acetyl, propionyl, malonyl or methylmalonyl groups. Following each condensation, FASs catalyze a complete reductive reaction cycle via ketoreduction, dehydration and enoylreduction of the β-keto group. In contrast, PKSs introduce structural diversity into their products by curtailing some of these reaction cycles. Termination of chain elongation may occur by thiolysis or acyl transfer.

The distinction between large multidomain enzymes and enzyme complexes composed of sets of separate freely dissociable proteins is reflected in the overall architecture of type I and type II enzymes, respectively. It appears that the architecture of discrete proteins (type II), capable of associating into a multienzyme complex, is appropriate for systems involving multiple repeated reaction cycles. The biosynthesis of a defined peptide product, however, primarily depends on the existence of a template containing the correct order of an appropriate number of substrate-activating (and modifying) modules [1–5,21]. So far, the peptide synthases studied, are exclusively the large multifunctional enzymes of modular arrangement (classified as type I [15–20]). Nevertheless, it is believed that each module is composed of distinct domains, which might be expected to retain their function if separated into individual enzymes [5,21,22].

We recently demonstrated that a core fragment composed of the first 656 residues of the peptide synthase involved in the biosynthesis of the cyclic decapeptide antibiotic gramicidin S, GrsA (which is similar to tyrocidine synthetase 1, TycA; see Fig. 1) restored all activities of the native peptide synthetase, with the exception of racemization [22]. A further carboxy-terminal deletion (100 amino

Figure 1



Schematic diagram of tyrocidine synthetase 1 (TycA) showing the relative location, extension and organization of specialized domains involved in substrate (phenylalanine)-dependent adenylation (red), thioester formation (green; PCP), and racemization (blue). Core motifs involved in these catalytic functions are indicated by boxes (1, LKAGGAYVPID; 2, YTSGTTGxPKG; 3, GELCIGGxGxARGY; 4, YxTGD; 5, VKIRGxRIELGEIE; 6, DNFYxLGGDSI; His, HHLVxDGISW; A, AYxTExNDLLLA; B, EGHGRExIIE; C, RTVGWFTSxYPVLLD; D, FNYLGQFD). The location and primary structure of the His_6 tagged PCP are displayed, and the 4′-PP binding site is shown in green letters.

Table 1

Enzyme	Organism	Core sequences
PCP (*tycA*)	*B. subtilis*	(58)- Y S L G G D S I Q A -(38)
FAS-ACP	*E. coli*	(30)- E D L G L D S L D T -(38)
FAS-ACP	*S. cerevisiae*	(75)- K D L G L D S L D T -(40)
PKS-ACP (*act*)	*S. coelicolor*	(35)- E D I G Y D S L A L -(41)
PKS-ACP (*gra*)	*S. violaceoruber*	(34)- E E L G Y D S L A L -(41)
PKS-ACP (*tcm*)	*S. claucescens*	(34)- D A L G Y D S L A L -(51)

TycA, tyrocidine synthetase 1; *act*, actinorhodin synthase; *gra*, granaticin synthase; *tcm*, tetracenomycin synthase. Serine residues shown in yellow are the binding site of 4′-PP.

acids) of this core fragment, which removes core motif 6 (LGGDS(L/I)) leads to an enzyme (domain) that has the primary structure of an adenylate-forming enzyme and that still shows a phenylalanine-dependent adenylation, but is unable to catalyze thioester formation. Therefore, this 100-residue domain was assumed to function as a peptidyl carrier protein (PCP), carrying the activated amino acids as thioesters tethered to 4′-PP [5,9,22]. FASs and PKSs also contain a functionally homologous protein or domain, the acyl carrier protein (ACP), that acts as an essential component of both synthases [31–37]. The basic function of this group of enzymes involves the transfer of an acyl group linked to the sulfhydryl residue of a covalently attached 4′-PP prosthetic cofactor. This family of proteins (ACPs and PCPs) shows high overall similarity around the 4′-PP attachment site ((L/I)GxDS(L/I); Table 1).

Here, we focus on the cloning and biochemical characterization of the putative PCP domain of TycA. The fact that we can show phosphopantetheinylation and covalent thioester binding of phenylalanine in PCP strongly supports both the multiple carrier model of non-ribosomal peptide biosynthesis and the peptidyl carrier activity of the thiolation domain. The thiolation domain can clearly function as a distinct enzyme, supporting the notion that peptide synthetases are arranged in modules. In addition, we demonstrate that a self-assembled module (type II synthetase), consisting of the adenylation domain of GrsA and the peptidyl carrier domain of TycA has activity *in vitro*.

Results and discussion

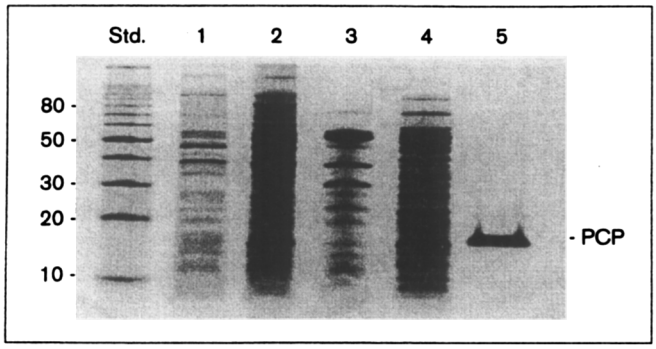
Cloning and expression of the TycA thiolation domain

To illustrate the biochemical features of a putative peptidyl carrier domain obtained from a distinct amino-acid-activating module, we have amplified and cloned the PCP coding region of TycA [38,39]. Primers for the polymerase chain reaction (PCR) were chosen after consideration of the primary structure of common ACPs involved in fatty acid or polyketide synthesis [31–37]. These ACPs are ~100 ± 25 amino acids long, with the active site serine residue generally located ~44 amino acids upstream of the carboxyl-terminus (Table 1). We also bore in mind the point at which the homology between exclusively adenylate-forming enzymes and peptide synthetases terminates, that is, ~50 amino acids upstream of the 4′-PP binding site (core motif 6; [17]). Previous dissection studies established that the thiolation domain is the carboxy-terminal region (~100-amino-acid extension) of an adenylation domain [22]. The coding region of the putative peptidyl carrier domain of TycA has been amplified and cloned (Fig. 1).

The primary structure of PCP and the location of the 4′-PP binding site are summarized in Figure 1 and Table 1. The thiolation domain is composed of 115 residues (including His_6-tag), and has a deduced molecular weight of 13.12 kDa. Interestingly, the isoelectric point and net charge (at pH 7.0) of PCP significantly deviates from that known for type II ACPs. For example, the corresponding ACP–His_6 fusion protein of *Escherichia coli* [35], which has 84 residues (molecular weight of 9.82 kDa), represents a small acidic (pI = 4.43) and anionic (net charge of –13.89, at pH 7.0) enzyme. In contrast, the thiolation domain of TycA is almost neutral, having a pI of 7.09 and a net charge of 0.2 (at pH 7.0). The significance of these differences remains unclear at present; however, it must be considered that the PCP (domain) is only a segment of a multifunctional protein, and normally acts in concert with other domains, not separately.

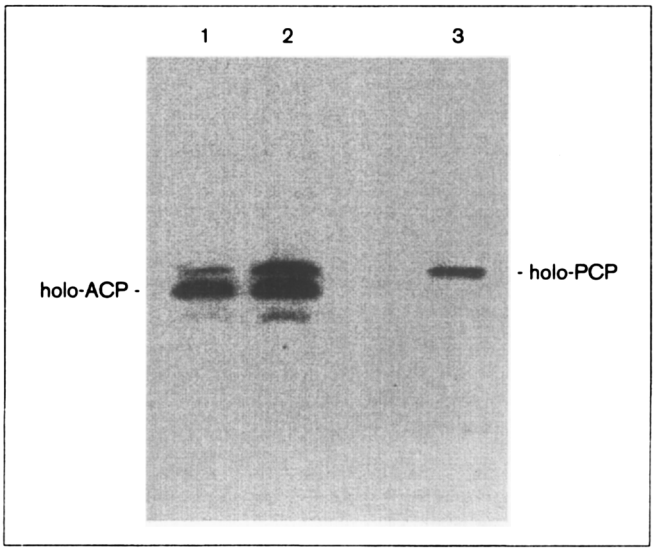
Amplification and cloning of the thiolation domain was performed as described in Materials and methods. The junctions between vector and insert DNA were sequenced to confirm the correct integration of *pcp* and to ensure that the insert was ligated in frame with the carboxy-terminal His_6 tag. The fermentation parameters (culture medium, temperature and isopropyl-β-D-thiogalactopyranoside (IPTG)-concentration) of the *E. coli* strain M15(pREP4/ pPCP-His)

Figure 2



Expression and purification of PCP. Samples were analyzed by 16.5 % SDS-PAGE (Materials and methods). The Coomassie-stained gel shows total cell extracts of *E. coli* M15(pREP4/pPCP-His) at time = 0 (lane 1) and 2 h after induction (lane 2). Cellular debris (lane 3) obtained after sonication of the cells was removed by centrifugation. The supernatant (lane 4) was directly applied to a Ni^{2+}-charged chelating agarose. PCP-containing fractions, obtained by applying a linear imidazole gradient, were pooled (lane 5), dialyzed and quantified.

Figure 3



β-[^{3}H]alanine labeling of recombinant PCP. The labeling of holo-PCP was performed in *E. coli* SJ16, which is defective in the biosynthesis of 4′-PP precursor β-alanine. The detailed procedure is described in Materials and methods. Samples (lane 1, crude cell extracts of *E. coli* SJ16(pPCP-His) at time = 0 and lane 2, 2 h after induction; lane 3, protein pool after affinity purification on chelating agarose) were applied to SDS-PAGE, and the label was identified by the autoradiography of the dried gel.

were first optimized. Using these conditions, a high-level expression was obtained for PCP as a predominantly soluble cytoplasmic protein (Fig. 2).

The cells overproducing PCP were resuspended in sonication buffer and lysed (Material and methods). After sonication and centrifugation the supernatant was adjusted to 30 mM imidazole and applied to a Ni^{2+}-charged chelating agarose, previously equilibrated with the same buffer. The addition of imidazole to the application buffer increased the stringency of the washes, considerably reducing unspecific binding on the column. When a linear gradient of imidazole was applied, PCP eluted within a single peak at a concentration of about 150 mM imidazole. Appropriate protein fractions were pooled, dialyzed against assay buffer, and the protein content was quantified. We purified the recombinant PCP to apparent homogeneity. SDS-polyacrylamide gel gave an apparent molecular mass of ~16 kDa, 3 kDa larger than expected. It is common for small proteins carrying His_6 tags to migrate more slowly on SDS gels than do equivalent untagged proteins.

Conversion of apo-PCP to holo-PCP

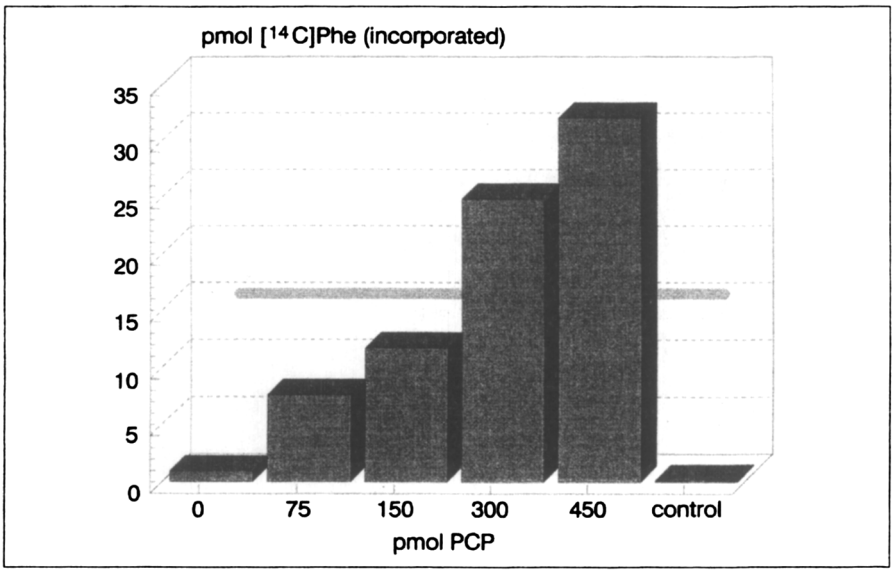
The most significant feature of ACPs and PCPs is their prosthetic group, 4′-PP [6–9,36,40] which is attached to an absolutely conserved serine residue (Table 1). The β-alanyl sidechain and the pantothenate portion of the 4′-PP moiety serve as a linker between the phosphodiester-ACP/PCP bond and the terminal cysteamine thiol residue, required for covalent binding of the activated substrate. This structure assists the prosthetic group's ability to function as a swinging arm, shuttling a growing acyl or peptidyl chain between miscellaneous active sites (e.g. between adjacent substrate-activating modules of a peptide synthetase during the sequential addition of up to 15 amino acids).

Recently, the enzyme catalyzing the phosphopantetheinylation of the ACP in *E. coli* (holo-ACP synthase; ACPS), has been cloned and characterized by Lambalot and Walsh [40,41]. Previous studies had revealed that several recombinant peptide synthetases expressed in *E. coli* were only partially pantetheinylated [8,22]. Until recently, nothing was known about the enzyme(s) that is presumably similar to ACPS, the specific 4′-PP-acyltransferase that catalyzes the posttranslational modification of peptide synthetases with the cofactor 4′-PP. A novel group of small proteins (~30 kDa), whose genes were previously shown to be associated with peptide antibiotic production [42–46], are now known to catalyze the modification of peptide synthetases from the apo- to the holo-enzyme (Lambalot *et al.* [47], this issue). In this context, co-expression of *pcp* with *gsp*, encoding the phosphopantetheinyl transferase Gsp associated with the gramicidin S biosynthetic operon, led to an increased production of holo-PCP (data not shown). This superfamily of phosphopantetheinyl transferases (Sfp, EntD, etc.), have been biochemically characterized by Lambalot *et al.* ([47], this issue).

To confirm the phosphopantetheinylation of recombinant PCP, we investigated the incorporation of β-[^{3}H]alanine into the overproduced protein. The plasmid pPCP-His

Figure 4

PCP-dependent thioester formation. The covalent incorporation of [^{14}C]phenylalanine was measured in the presence of a constant 150 pmol of the phenylalanine-specific adenylation domain PheA, and increasing concentrations of the thiolation domain PCP (0–3 mole equivalents). The PCP control reaction was performed in the presence of 150 pmol PCP without PheA. The vertical bars represent the amount of covalently bound, acid-stable label (in pmol). The horizontal line corresponds to the value obtained with 150 pmol of PheAT, comprising both the adenylation and the thioester forming domain of GrsA.



was used to transform the *E. coli* strain SJ16 [8,36], which is a *panD* mutant defective in the biosynthesis of β-alanine, a precursor of pantothenate (Fig. 6). After β-alanine starvation, cells were used to inoculate a minimal medium supplemented with [^{3}H]-labeled β-alanine. Expression and purification of the thiolation domain were performed as previously described. Total cellular extracts before and after induction were analyzed by SDS-PAGE (as was affinity-purified PCP) (Fig. 3).

Figure 3 reveals the consecutive expression of a 10 kDa protein (lane 1), most likely the holo-ACP of the host. A similar amount of a slightly larger protein (16 kDa) was also observed after a 2 h induction with IPTG (lane 2). After affinity purification using chelate chromatography, the 16 kDa protein was only detected in the eluant (lane 3), clearly defining this protein as the His_6-tagged PCP. Small quantities of this protein are present before induction (lane 1) indicating that the T5 promoter controlling *pcp* expression was slightly leaky. The amounts of labeled holo-ACP and holo-PCP, observed by SDS-PAGE, are similar; however, the comparative analysis of the amount of radiolabeled and expressed PCP clearly verifies that only a small portion of the thiolation domain was modified (data not shown). This could be explained by a low concentration of modifying enzyme(s), insufficient to alter the entire ACP and PCP pool expressed, or by assuming that apo-PCP is a poorer substrate for *E. coli* 4′-PP-transferase(s). Lambalot *et al.* [47] (this issue) have shown that ACPS modifies ACP of *E. coli*, whereas a different enzyme, EntD, modifies EntF and PCP *in vitro*. Since ACPS has no affinity to PCP, the observed conversion of apo- to holo-PCP should be attributed to EntD activity. Nevertheless, our results clearly demonstrate that β-[^{3}H]-alanine is incorporated into PCP, modifying it from the apo- to the holo-protein. Post-translational modification is also observed by mass spectroscopy; recombinant PCP has a molecular weight of 13.13 kDa for the apo protein and 13.431 kDa for the holo protein [47]. This might indicate that PCP should be a functional enzyme, which is most likely to act as an amino acyl or a peptidyl carrier during non-ribosomal peptide synthesis.

Acylation of holo-PCP

The substrate activation during non-ribosomal peptide synthesis includes the covalent binding of the carboxyl-activated amino acid as a thioester via the enzyme-linked cofactor 4′-PP [1–5]. Recently, we reported on the functional dissection of the peptide synthetase GrsA, involved in the biosynthesis of the cyclic decapeptide antibiotic gramicidin S in *Bacillus brevis* ATCC 9999 [22]. Here, we describe two deletion mutants: PheA, composed of the primary structure of a solely adenylate-forming enzyme but lacking the 4′-PP attachment site and therefore incapable of covalently binding the substrate, and PheAT [22], which retains the thiolation domain (PCP) as a carboxy-terminal extension of the adenylation domain mentioned above restoring its ability to form the thioester bond. These results confirmed that PCP functions as an amino acyl and peptidyl carrier during the directed synthesis of a peptide product from a protein template [5,9].

To investigate the enzymatic role of PCP as an independent amino acyl or peptidyl carrier protein, we have performed the thioester formation assay (Materials and methods) using a mixture of the adenylation domain (PheA) of GrsA and PCP. In this reaction, PheA should catalyze the formation of an amino acyladenylate. This

relatively unstable intermediate should then be stabilized by thioesterification on the prosthetic 4′-PP group of PCP. No thioester formation was detectable when only PheA is present, as expected (Fig. 4). The low level of [^{14}C]phenylalanine bound to PheA is in good agreement with values measured for mutants that were incapable of covalent binding of the substrate amino acid, for example when the active site serine residue of core motif 6 was mutated to alanine [8,22]. The addition of low concentrations of PCP (75 pmol) resulted in a significant, 10-fold increase in [^{14}C]phenylalanine incorporation; however, the amount of covalently bound, acid-stable label constantly increased with the amount of PCP available. Most notably, an equimolar ratio (150 pmol) of PheA and PCP, in effect representing a multienzyme type II variant of PheAT, gave approximately the same level of [^{14}C]phenylalanine incorporation (~80 %) as did the undissected bifunctional PheAT enzyme (Fig. 4). The nature of the thioester linkage was confirmed by its stability to acid treatment and its susceptibility to base hydrolysis [8,22,48]. Moreover, PCP contains no cysteine residues (Fig. 1), indicating that the thiol group of the 4′-PP cofactor is the only possible thioester binding site on the holo enzyme.

These results clearly indicate that PCP is involved in covalent thioester binding of the activated substrate amino acid in peptide synthesis. To confirm that the label is on PCP not PheA, we have separated the reaction mixtures by SDS-PAGE. Some of the label remained associated with the adenylation domain even in the absence of PCP (Fig. 5). But addition of PCP to the reaction mixture caused a new signal, corresponding to PCP, to appear, and did not increase the amount of label associated with PheA. Taken together with the results of the thioester formation assay, this confirms that PCP is indeed aminoacylated. The scheme shown in Figure 6 summarizes the results obtained on PCP posttranslational modification and acylation.

Significance

This report presents the characterization of PCP, the thiolation domain of TycA, an enzyme involved in the biosynthesis of the cyclic decapeptide antibiotic tyrocidine [38,39]. It is believed that many of the enzymes that synthesize this type of compound are composed of modules that can function essentially independently. We have cloned the DNA that encodes the PCP domain and overproduced a tagged version of the domain in the absence of the other domains of the TycA protein. The isolated domain can be modified with phosphopantetheine, indicating that it belongs to the class of ACPs involved in the transfer of acyl intermediates during the biosynthesis of fatty acids and polyketides. The detection of phosphopantetheine-modified PCP supports the idea that the enzyme may act as a peptidyl carrier, shuttling the growing peptide chain between the different modules of a protein template during the directed and sequential synthesis of the corresponding peptide product, which remains 'tethered' to the PCP domain throughout the process.

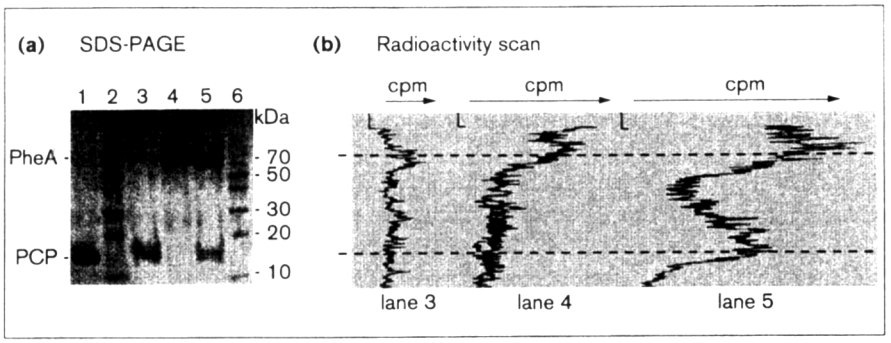
To determine the ability of holo-PCP to carry a particular acyl group, we have investigated its phenylalanylation in the presence of an adenylation domain (PheA; [22]). The detection of a radiolabel covalently attached to PCP after coincubation with PheA shows that holo-PCP can be acylated. Moreover, this experiment shows that the catalytic domains of peptide synthetases are able to act not only as independent enzymes but also in *trans* with other functional domains. These findings may provide a tool to construct peptide synthetases that are formed of several independent enzymes, greatly increasing our ability to generate peptides with diverse structures.

Material and methods

PCR amplification and DNA manipulations

PCR amplification of the *tyc*A fragment encoding the thiolation domain (PCP) has been used to generate the terminal restriction sites for the subsequent cloning, and was performed using Deep VentR® DNA polymerase and 10 × reaction buffer from New England Biolabs (Schwalbach, Germany) following the manufacturer's protocol. The sequences of the 5′-modified oligonucleotides used were as follows (italicized, modified sequences; bold, restriction sites): oligo 5′-PCP-*Nco*I: 5′-*AAC* **CCA TGG** ACA AAA TGC CGC TTA CGC CA-3′; and 3′-PCP-*Bgl*II: 5′-*CCT* ***AGA*** **TCT** CGT CGT GCT CTT GAC AAA AAG-3′.

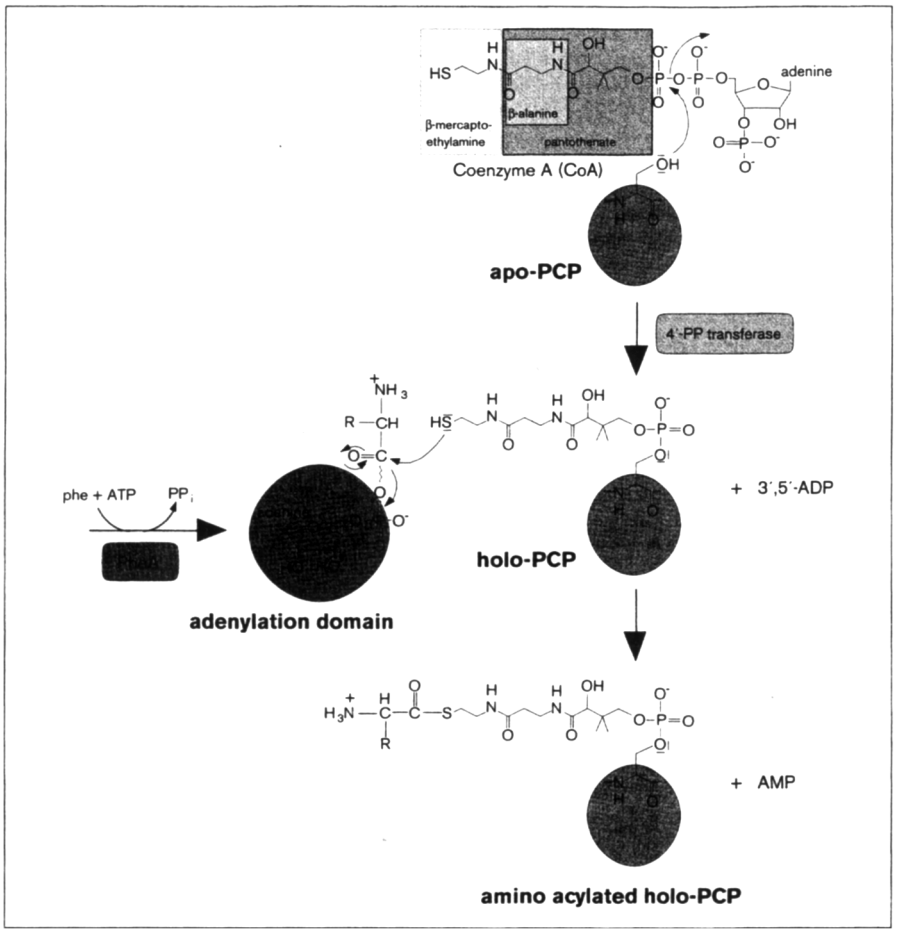
Figure 5



Determination of PCP acylation. The thioester formation assay was performed as described [22]. The mixtures were quenched with TCA, and the precipitated proteins were redissolved in TE buffer, neutralized and applied to SDS-PAGE. The gel was analyzed by Coomassie-staining, and the label was identified by scanning. The samples are: lane 1, PCP-standard; lanes 2 and 6, 10 kDa ladder; and three radioassays containing lane 3, 300 μM PCP; lane 4, 150 μM PheA and lane 5, 300 pmol PCP plus 150 pmol PheA.

Figure 6

Scheme showing the modification of the PCP with the 4′-PP moiety of coenzyme A (CoA) and PCP acylation in the presence of an adenylation domain (orange circle). A 4′-PP transferase catalyzes the transfer of the prosthetic group from CoA to Ser-68 of apo-PCP (green circle). The holo-PCP can then stabilize activated amino acyl groups through a thioester linkage to the terminal cysteamine residue of the cofactor. Premature substrate activation by an adenylation domain, for example PheA (orange), is indispensable for the subsequent formation of the covalent thioester linkage. The β-alanyl moiety within the pantothenate residue is emphasised in light orange (compare with Fig. 3: β-[^{3}H]alanine incorporation into recombinant PCP).



We used the pQE60 His$_6$ tag fusion vector, purchased from Qiagen (Hilden, Germany), to clone the truncated *tyc*A(PCP) fragment. Standard procedures were used for the digestion with restriction enzymes, cloning of the DNA fragments and the preparation of recombinant plasmid DNA [49]. We also confirmed the fusion sites between the vector and the *pcp* gene by sequencing, using the chain termination method of Sanger *et al.* [50]. Sequence runs were performed on an ABI prism 310 Genetic Analyzer. Sequences of the primers used were as follows: oligo 5′-promoter: 5′-GGC GTA TCA CGA GGC CC-3′; and 3′-His tag: 5′-ACG CCC GGC GGC AAC CG-3′.

Expression and purification of the His$_6$-tagged PCP

The pQE60-derivative containing the amplified *pcp* (designated pPCP-His) was transformed in *E. coli* strain M15(pREP4) and streaked out on 2 × YT plates containing 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin [51]. After overnight incubation at 37° C the colonies were resuspended from the plates, and the suspension obtained was directly used to inoculate 2 × YT medium supplemented with kanamycin (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}) and magnesium chloride (20 mM). Cells were grown at 30° C with moderate shaking until A_{600} nm reached 0.8. IPTG (1 mM) was added and cultures were grown for an additional 2 h. Expression was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 16.5 %) using the method of Schägger and von Jagov [52].

Further operations, except for fast performance liquid chromatography (FPLC), were carried out at 4° C. Cells were harvested by centrifugation for 15 min at 6000 × g and resuspended (3 ml g^{-1} wet weight) in sonication buffer (50 mM HEPES, 300 mM sodium chloride, pH 7.8). Subsequently, the cells were lysed by freezing, thawing and sonication using a Branson Sonifier at middle output. Cellular debris was removed by centrifugation at 40 000 × g for 30 min, and the supernatant obtained was adjusted to 30 mM imidazole and directly applied to a Ni^{2+}-charged chelating agarose (Ni-NTA resin from Qiagen; Hilden, Germany), previously equilibrated with the same buffer. Using a FPLC system purchased from Pharmacia (Freiburg, Germany), the proteins were eluted by applying an imidazole gradient ranging over 30–300 mM imidazole in sonication buffer. Samples were analyzed by SDS-PAGE, pooled, dialyzed against assay buffer (50 mM HEPES pH 7.8, 100 mM sodium chloride, 10 mM magnesium chloride, 2 mM dithioerythritol and 1 mM EDTA) and quantified using the procedure of Bradford [53].

β-[^{3}H]alanine incorporation

E. coli strain SJ16 (*pan*D), a β-alanine auxotroph [36], was transformed with pPCP-His, maintained for 36 h at 37° C on agar plates with M9 minimal medium supplemented with 10 μg ml^{-1} tetracycline and 50 μg ml^{-1} ampicillin. A single colony was used to inoculate 1 ml minimal medium E supplemented with 0.5 % D-glucose, 3 μM thiamine, 130 μM methionine, 10 μM β-alanine and 50 μg ml^{-1} ampicillin. Cells were grown overnight at 37° C, harvested and washed four times in 500 μl medium E (without β-alanine) to remove any traces of unbound β-alanine [8,36].

Labeling of PCP was performed in 10 ml of medium E containing all supplements including β-[^{3}H]alanine (purchased from Amersham, Braunschweig, Germany) at a final concentration and specific activity of 0.55 μM and 90.9 Ci mmol^{-1}, respectively [8,36]. This medium was

inoculated using 100 µl washed cells. Further expression and purification of the labeled PCP was performed as described above. Total cell extracts and samples from the chelate chromatography were analyzed by SDS-PAGE. The Coomassie-stained gel was soaked in Amplify (Amersham, Braunschweig, Germany) and dried. The label was identified by autoradiography on X-ray film (Kodak X-OMAT AR, Rochester, USA) and by scanning on a Berthold LB2723 thin-layer scanner II.

Thioester formation and cleavage

Covalent binding of phenylalanine by the recombinant PCP domain was tested as described previously [8,22]. Each assay mix contained 0 or 150 pmol of the adenylation domain PheA, 0–450 pmol of PCP, 2 mM ATP and 3 µCi (474 mCi mmol^{-1}) of [^{14}C]phenylalanine (purchased from Amersham, Braunschweig, Germany) in a total volume of 100 µl assay buffer. The reactions were allowed to proceed for 30 min at 37° C, and were stopped by the addition of 2 ml chilled 7 % trichloroacetic acid (TCA) followed by a further incubation on ice for 30 min. TCA-precipitated protein was collected on glass fibre filter (GF-92; Schleicher & Schuell, Dassel, Germany) and washed with an excess of TCA. After addition of 5 ml scintillation mixture (Rotiszint Eco Plus; Roth, Karlsruhe, Germany) the enzyme-bound label was quantified using a 1900CA Tri-Carb liquid scintillation analyzer (Packard).

Alternatively, the radioassay was quenched with 700 µl 10 % TCA and 3 µl bovine serum albumin (25 mg ml^{-1}). Subsequently, the precipitated proteins were resolved in 40 µl TE buffer, neutralized by the addition of about 5 µl 1 M Tris/HCl (pH 7.5) and applied to SDS-PAGE. The Coomassie-stained gel was soaked in Amplify and analyzed as outlined above. The cleavage of the thioesterified phenylalanine was performed as described by Ullrich *et al.* [48].

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References

1. Zuber, P., Nakano, M.M. & Marahiel, M.A. (1993). Peptide antibiotics. In *Bacillus subtilis and other Gram-positive bacteria.* (Sonenshein, A.L., Hoch, J.A. & Losick, R. eds), pp. 897–916, *Am. Soc. Microbiol.*, Washington, DC, USA.
2. Marahiel, M.A. (1992). Multidomain enzymes involved in peptide synthesis. *FEBS Lett.* **307**, 40–43.
3. Kleinkauf, H. & von Döhren, H. (1996). A nonribosomal system of peptide biosynthesis. *Eur. J. Biochem.* **236**, 335–351.
4. Stachelhaus, T. & Marahiel, M.A. (1995). Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEMS Microbiol. Lett.* **125**, 3–14.
5. Stachelhaus, T., Schneider, A. & Marahiel, M.A. (1996). Engineered biosynthesis of peptide antibiotics. *Biochem. Pharmacol.* **52**, 177–186.
6. Stein, T., *et al.*, & Morris, H.R. (1994). Detection of 4′-phosphopantetheine at the thioester binding site for L-valine of gramicidin S synthetase 2. *FEBS Lett.* **340**, 39–44.
7. Stein, T., Kluge, B., Vater, J., Franke, P., Otto, A. & Wittmann-Liebold, B. (1995). Gramicidin S synthetase 1 (phenylalanine racemase), a prototype of amino acid racemases containing the cofactor 4′-phosphopantetheine. *Biochemistry* **34**, 4633–4642.
8. Gocht, M. & Marahiel, M.A. (1994). Analysis of core sequences in the D-phe activating domain of the multifunctional peptide synthetase TycA by site-directed mutagenesis. *J. Bacteriol.* **176**, 2654–2662.
9. Stein, T., *et al.*, & Morris, H.R. (1996). The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic enzymes. *J. Biol. Chem.* **271**, 15428–15435.
10. Krause, M., Marahiel, M.A., von Döhren, H. & Kleinkauf, H. (1985). Molecular cloning of an ornithine-activating fragment of the gramicidin S synthetase 2 gene from *Bacillus brevis* and its expression in *Escherichia coli*. *J. Bacteriol.* **162**, 1120–1125.
11. Skarpeid, H.J., Zimmer, T.-L., Shen, B. & von Döhren, H. (1990). The proline-activating activity of the multienzyme gramicidin S synthetase 2 can be recovered on a 115 kDa tryptic fragment. *Eur. J. Biochem.* **187**, 627–633.
12. Skarpeid, H.J., Zimmer, T.-L. & von Döhren, H. (1990). On the domain construction of the multienzyme gramicidin S synthetase 2. *Eur. J. Biochem.* **189**, 517–522.
13. Hori, K., *et al.*, & Saito, Y. (1991). The nucleotide sequence for a proline-activating domain of gramicidin S synthetase 2 gene from *Bacillus brevis*. *J. Biochem.* **110**, 111–119.
14. Kurotsu, T., Hori, K., Kanda, M. & Saito, Y. (1991). Characterization and location of the L-proline activating fragment from the multifunctional gramicidin S synthetase 2. *J. Biochem.* **109**, 763–769.
15. Weber, G., Schörgendorfer, K., Schneider-Scherzer, E. & Leitner, E. (1994). The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. *Curr. Genet.* **26**, 120–125.
16. Haese, A., Schubert, M., Herrmann, M. & Zocher, R. (1993). Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalyzing *N*-methyldepsipeptide formation in *Fusarium scirpi*. *Mol. Microbiol.* **7**, 905–914.
17. Turgay, K., Krause, M. & Marahiel, M.A. (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.
18. Scott-Craig, J.S., Panaccione, D.G., Pocard, J.-A. & Walton, J.D. (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-kilobase open reading frame. *J. Biol. Chem.* **267**, 26044–26049.
19. Cosmina, P., Rodriguez, F., de Ferra, F., Perego, M., Venema, G. & van Sinderen, D. (1993). Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. *Mol. Microbiol.* **8**, 821–831.
20. Fuma, S., *et al.*, & Yamane, K. (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.
21. Stachelhaus, T., Schneider, A. & Marahiel, M.A. (1995). Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science* **269**, 69–72.
22. Stachelhaus, T. & Marahiel, M.A. (1995). Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. *J. Biol. Chem.* **270**, 6163–6169.
23. de Crécy-Lagard, V., Marlière, P. & Saurin, W. (1995). Multienzymatic non-ribosomal peptide biosynthesis: identification of the functional domains catalyzing peptide elongation and epimerization. *Life Sci.* **318**, 927–936.
24. Tokita, K., Hori, K., Kurotsu, T., Kanda, M. & Saito, Y. (1993). Effect of single base substitutions at glycine-870 codon of gramicidin S synthetase 2 gene on proline activation. *J. Biochem.* **114**, 522–527.
25. Pavela-Vrancic, M., Pfeifer, E., Schröder, W., von Döhren, H. & Kleinkauf, H. (1994). Identification of the ATP-binding site in tyrocidine synthetase 1 by selective modification with fluorescin 5′-isothiocyanate. *J. Biol. Chem.* **269**, 14962–14966.
26. Pavela-Vrancic, M., Pfeifer, E., van Liempt, H., Schäfer, H.-J., von Döhren, H. & Kleinkauf, H. (1994). ATP binding in peptide synthetases: determination of contact sites of the adenine moiety by photoaffinity labeling of tyrocidine synthetase 1 with 2-azidoadenosine triphosphate. *Biochemistry* **33**, 6276–6283.
27. Pavela-Vrancic, M., van Liempt, H., Pfeifer, E., Freist, W. & von Döhren, H. (1994). Nucleotide binding by multienzymatic peptide synthetases. *Eur. J. Biochem.* **220**, 535–542.
28. Wakil, S.J. (1989). Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* **28**, 4523–4530.
29. Hopwood, D.A. & Sherman, D.H. (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu. Rev. Genet.* **24**, 37–66.
30. Hopwood, D.A. & Khosla, C. (1992). Genes for polyketide secondary metabolic pathways in microorganisms and plants. In CIBA Foundation Symposium No. 171 on Secondary Metabolites: Their Function and Evolution. pp. 88–112, Wiley, Chichester, UK.
31. Khosla, C., Ebert-Khosla, S. & Hopwood, D.A. (1992). Targeted gene replacement in a *Streptomyces* polyketide synthase gene cluster: role for the acyl carrier protein. *Mol. Microbiol.* **6**, 3237–3249.
32. Bibb, M.J., Biro, S., Motamedi, H., Collins, J.F. & Hutchinson, C.R. (1989). Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI* genes provides key information about the enzymology of polyketide antibiotic biosynthesis. *EMBO J.* **8**, 2727–2736.

33. Fernadez-Moreno, M.A., Martinez, E., Caballero, J.L., Ichinose, K., Hopwood, D.A. & Malpartida, F. (1994). DNA sequence and functions of the *act*VI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). *J. Biol. Chem.* **269**, 24854–24863.
34. Sherman, D.H., Malpartida, F., Bibb, M.J., Kieser, H.M., Bibb, M.J. & Hopwood, D.A. (1989). Structure and deduced function of the granaticin producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tu22. *EMBO J.* **8**, 2717–2725.
35. Vanaman, T.C., Wakil, S.J. & Hill, R.L. (1968). The complete amino acid sequence of the acyl carrier protein of *Escherichia coli*. *J. Biol. Chem.* **243**, 6420–6431.
36. Rock, C.O., Cronan, J.E. & Armirage, I.M. (1981). Molecular properties of acyl carrier protein derivatives. *J. Biol. Chem.* **256**, 2669–2674.
37. Cheret, G., Mattheakis, L.C. & Sor, F. (1993). DNA sequence analysis of the YCN2 region of chromosome XI in *Saccharomyces cerevisiae*. *Yeast* **9**, 661–667.
38. Marahiel, M.A., Krause, M. & Skarpeid, H.-J. (1985). Cloning of the tyrocidine synthetase 1 gene from *Bacillus brevis* and its expression in *Escherichia coli*. *Mol. Gen. Genet.* **201**, 231–236.
39. Weckermann, R., Fürbaß, R. & Marahiel, M.A. (1988). Complete nucleotide sequence of the *tyc*A gene coding for the tyrocidine synthetase 1 from *Bacillus brevis*. *Nucleic Acids Res.* **16**, 11841.
40. Lambalot, R.H. & Walsh, C.T. (1995). Cloning, overproduction, and characterization of the *Escherichia coli* holo-acyl carrier protein synthase. *J. Biol. Chem.* **270**, 24658–24661.
41. Lambalot, R.H. & Walsh, C.T. (1996). Holo-acyl carrier protein synthase of *Escherichia coli*. *Meth. Enzymol*, in press.
42. Armstrong, S.K., Pettis, G.S., Forrester, L.J. & McIntosh, M.A. (1989). The *Escherichia coli* enterobactin biosynthesis gene, *ent*D: nucleotide sequence and membrane localization of its protein product. *Mol. Microbiol.* **3**, 757–766.
43. Borchert, S., Stachelhaus, T. & Marahiel, M.A. (1994). Induction of surfactin production in *Bacillus subtilis* by *gsp*, a gene located upstream of the gramicidin S operon in *Bacillus brevis*. *J. Bacteriol.* **176**, 2458–2462.
44. Nakano, M.M., Marahiel, M.A. & Zuber, P. (1988). Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J. Bacteriol.* **170**, 5662–5668.
45. Nakano, M.M., Corbell, N., Besson, J. & Zuber, P. (1992). Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Mol. Gen. Genet.* **232**, 313–321.
46. Gaidenko, T.A., Belitsky, B.R. & Haykinson, M.J. (1988). Characterization of a new pleuotropic regulatory gene from *Bacillus licheniformis*. *Biotechnologia* **3**, 13–19.
47. Lambelot, R.H., *et al.*, & Walsh, C.T. (1996). A new enzyme superfamily–the phosphopantetheinyl transferases. *Chemistry & Biology* **3**, 923–936.
48. Ullrich, C., Kluge, B., Palacz, Z. & Vater, J. (1991). Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*. *Biochemistry* **30**, 6503–6508.
49. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, NY, USA.
50. Sanger, F., Miklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
51. Zamenhof, P.J. & Villarejo, M. (1972). Construction and properties of *Escherichia coli* strains exhibiting α-complementation of β-galactosidase fragments *in vivo*. *J. Bacteriol.* **107**, 171–178.
52. Schägger, H. & von Jagov, G. (1987). Tricine-sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379.
53. 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.