Streptothricin Biosynthesis Is Catalyzed by Enzymes Related to Nonribosomal Peptide Bond Formation

MIGUEL A. FERNÁNDEZ-MORENO,1 CARLOS VALLÍN,2 AND FRANCISCO MALPARTIDA1*

Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain and Centro de Química Farmacéutica, Atabay, Havana, Cuba

Received 27 March 1997/Accepted 15 August 1997

In a search for strains producing biocides with a wide spectrum of activity, a new strain was isolated. This strain was taxonomically characterized as Streptomyces rochei F20, and the chemical structure of the bioactive product extracted from its fermentation broth was determined to be a mixture of streptothricins. From a genomic library of the producer strain prepared in the heterologous host Streptomyces lividans, a 7.2-kb DNA fragment which conferred resistance to the antibiotic was isolated. DNA sequencing of 5.2 kb from the cloned fragment revealed five open reading frames (ORFs) such that ORF1, -2, -3, and -4 were transcribed in the same direction while ORF5 was convergently arranged. The deduced product of ORF1 strongly resembled those of genes involved in peptide formation by a nonribosomal mechanism; the ORF2 product strongly resembled that of mphA and mphB isolated from Escherichia coli, which determines resistance to several macrolides by a macrolide 2’-phosphotransferase activity; the ORF3 product had similarities with several hydrolases; and the ORF5 product strongly resembled streptothricin acetyltransferases from different gram-positive and gram-negative bacteria. ORF5 was shown to be responsible for acetyl coenzyme A-dependent streptothricin acetylation. No similarities in the databases for the ORF4 product were found. Unlike other peptide synthases, that for streptothricin biosynthesis was arranged as a multifunctional protein. Insertional inactivation of ORF1 and ORF2 (and to a lesser degree, of ORF3) abolishes antibiotic biosynthesis, suggesting their involvement in the streptothricin biosynthetic pathway.

During a screening process, a streptomycte producer of broad-range antibiotic activity was isolated from soil samples; this strain has been taxonomically characterized as Streptomyces rochei F20, and its active compound has been described as a mixture of streptothricins (mostly F and traces of D and E) (41a). The present paper describes the isolation, DNA sequence, and partial characterization of the biosynthetic pathway for streptothricin produced by this newly isolated strain. Streptothricin was one of the first actinomycete antibiotics to be described (56). It has a broad spectrum, with antibacterial as well as antifungal activity. Its chemical elucidation was described by Kusumoto and coworkers (30) (Fig. 1). It contains a heterocyclic β-amino acid (streptolidine), an amino sugar (4-carbamido-D-gulosamine), and, by amide linkage at C-2, a β-lsine chain which varies from one to six units in streptothricins F to A, respectively, and which includes seven units for streptothricin X (22, 31). More recently, other members of the family, which are chemically closely related to streptothricin, have been described (1, 23, 25).

Members of the streptothricin family have, in addition to a potent inhibitory activity for prokaryotic protein synthesis (11), cytotoxicity which prevents their clinical or veterinary use. However, this may well be useful in situations in which toxicity may not be a problem, such as in agriculture or prevention of microbial contamination, etc. In the same manner, it has been used as a selection marker in recombinant DNA work against various organisms, including fungi and bacteria (10, 27).

Isolation of streptothricin resistance genes, but not biosynthetic ones, from different gram-positive and gram-negative bacteria has been described previously, and it was shown that the resistance mechanism is due to a modification of the antibiotic by acetylation at one amino group of the β-lysine by transacetylation from acetyl coenzyme A (acetyl-CoA) (53).

MATERIALS AND METHODS

Bacterial strains. The Escherichia coli strains were JM101 and JM110 (58). The Streptomyces lividans 66 strain was TK21 (str-6 SLP2′ SLP3″) (15). The Streptomyces albus strain was 31074 (6). As a streptothricin producer strain, Streptomyces lavendulae ATCC 8664 was used. For testing sensitivity to antibiotics, Micrococcus luteus ATCC 10240 was used. The fungal strain was Penicillium chrysogenum ATCC 10003.

Taxonomic characterization. The microbial identification of the newly isolated streptothricin-producing strain was carried out at Analytical Services, Inc. (Wilmington, Vt.), by high-resolution gas chromatography with the MIDI/Hewlett-Packard Microbial Identification System. The strain was identified as S. rochei with a similarity index of 0.6; because the similarity index indicates there is not a complete identity, we suggest the name S. rochei F20 for our strain.

Plasmids and bacteriophages. The E. coli plasmids were plUC19 (58), plJ2925 and plJ2921 (20), and pSU21 (2). E. coli M13-derivative phages mp18 and mp19 (58) were used for DNA sequencing. The Streptomyces plasmid vector was the high-copy-number plJ702 (21). The Streptomyces phage vector was the φC31-derivative KC515 (43).

Media, culture conditions, and microbiological procedures. Streptomyces manipulations were as described previously (15). For testing antibiotic production, several different culture media for growing S. rochei F20 were tried; eventually, liquid cultures were made in FM medium (1% yeast extract, 1% Bacto Tryptone, 25 mM TES buffer [pH 7.5]) at 30°C with good aeration. For selection of resistance, solid FM medium was supplemented with the streptothricin mixture obtained from the fermentation broth of S. rochei F20 grown until stationary phase. The broth was filtered, lyophilized, and 10-fold concentrated and 1,000-fold dilution of this was used.

Thioestrepton (Calbiochem; catalog no. 598226) was used at concentrations of 50 μg/ml in agar medium and 10 μg/ml in broth cultures. E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth (34). The range of action of S. rochei F20 biocide was tested by placing 0.5-cm circular plugs from an R5 plate on a plate (1.5% agar) overlaid with soft agar (0.6% agar) containing the possible sensitive organism to be checked. For gram-negative E. coli, the medium was LB (34); for gram-positive S. lividans, the medium was DNA (15); for M. luteus, the medium was LB; and for the fungus P. chrysogenum, the medium was MPDA (2% malt extract, 2% glucose, 0.1% Bacto...
Crude extracts were obtained after centrifugation at 14,926 x g; four cycles of 15 s each were applied. The filtrate was spotted on a 9-mm-diameter cellulose disk and assayed by adding 40 ml of this solution was incubated with approximately 40 ml of total DNA from S. rochei F20, partially digested with Sall3AI, was constructed on plasmid pIJ702 previously digested with BglII and with S. lividans TK21 as the host. The transformants were harvested and plated onto FM solid medium containing thio/streptomycin and the streptothricin mixture extracted from the fermentation broth.

Plasmid DNAs obtained from several resistant colonies were shown to contain a 7.2-kb DNA fragment which was clearly shown to be responsible for resistance to streptothricin when it was retransformed on the sensitive strains S. lividans and S. albus. Southern blot analysis confirmed that the cloned DNA fragment was present in the S. rochei F20 chromosome without rearrangement, while no hybridization was observed with DNA from the strain used as the cloning host.

DNA manipulations. Isolation, cloning, and manipulation of nucleic acids were as described for Streptomyces (15) and for E. coli (34).

**RESULTS**

**Biocide production and physicochemical properties.** To check antibiotic production by S. rochei F20 by the agar plug method, a series of solid media were used; R5 was shown to be the optimal medium for production. Activity was measured as the diameter of the growth inhibition halo for each tested strain. For S. lividans and M. luteus, a marked inhibition effect was observed, and for P. chrysogenum and E. coli the halos were smaller but still significant. Antibiotic production from liquid medium was optimal with FM medium under our experimental conditions. The time course for antibiotic production is that of a typical secondary metabolite of Actinomycetes. Antibiotic was produced during growth at 30°C, and it was maintained during the time tested (several hours into the stationary phase); no antibiotic production was detected when growth was carried out at 37°C.

The physicochemical properties of the antibiotic in the culture broth are similar to those well-established for the streptothricin family: solubility in water, thermostability, resistance to low pH, and sensitivity to alkaline conditions.

**Cloning and localization of the resistance gene.** To gain access to the biosynthetic genes for streptothricin production, we attempted to isolate the streptothricin resistance gene to which they might well be linked. Thus, a library of total DNA from S. rochei F20, partially digested with Sall3AI, was constructed on plasmid pIJ702 previously digested with BglII and with S. lividans TK21 as the host. The transformants were harvested and plated onto FM solid medium containing thio/streptomycin and the streptothricin mixture extracted from the fermentation broth.

Plasmid DNAs obtained from several resistant colonies were shown to contain a 7.2-kb DNA fragment which was clearly shown to be responsible for resistance to streptothricin when it was retransformed on the sensitive strains S. lividans and S. albus. Southern blot analysis confirmed that the cloned DNA fragment was present in the S. rochei F20 chromosome without rearrangement, while no hybridization was observed with DNA from the strain used as the cloning host.

The DNA fragment carrying the resistance gene was narrowed down within the cloned 7.2-kb fragment. With a set of overlapping restriction fragments (Fig. 2), a 1.45-kb SphiI fragment (of which 165 bp were from the vector) was shown to confer resistance to streptothricin.

**Sequence analysis of the resistance gene.** DNA sequencing was started at the unique EspI site toward the right-hand end of the resistance-inducing fragment and was extended for 5.2 kb to the left to a Sall site. Computer-assisted analysis of the sequenced region revealed five ORFs (Fig. 2), which were named (from left to right) ORF1, ORF2, ORF3, ORF4, and ORF5; the first four ORFs were transcribed rightward, whereas ORF5, the putative resistance gene, ran convergently from the rightmost end toward the other four ORFs.

The translation start point for each ORF was tentatively located by several criteria (57): (i) the overall distribution of GC content in the third position of the codons, (ii) the codon usage within the putative coding sequences, (iii) the observed similarities between the putative ORF product and those of other genes in the databases, and (iv) the presence of a canonical ribosome binding site (RBS) at a suitable distance from the putative translation start codon. This last criterion was considered for assignment of the ORF3 translation start codon; in contrast, ORF2, ORF4, and ORF5 lacked suitable RBS sequences for any of the putative translation start codons.

The ORF1 stop codon overlaps with the putative ORF2 start codon (ATGA), as do ORF2 and ORF3 (GTGA), suggesting...
translational coupling, as in many other Streptomyces genes (9, 48). The ORF4 start codon is assigned 24 bp downstream of the ORF3 stop codon. All of these data suggest the possibility of a polycistronic mRNA for all four ORFs. The ORF4 stop codon would be eight amino acids within ORF5, so that the carboxy-terminal regions of both ORFs would overlap.

The most relevant features of this region, which were deduced from its DNA sequence, are summarized in Table 1.

The whole sequence shows a G+C content (72.4%) typical for Streptomyces. However, DNA sequences upstream from the ORF1 and ORF5 start codons are richer in A+T (39.2 and 33%, respectively, instead of the typical 27.6%). These findings could suggest a regulatory role for these regions, which may well include the special promoter sequences for secondary metabolism genes and, therefore, may be subject to temporal control. Possibly noteworthy are several direct repeats, 19 to 20 bp in length and G+C rich, which are located immediately downstream from the ORF2 stop codon.

Deduced functions of the cloned genes. Searching databases for similarities with the cloned gene products revealed resemblances for some of them.

(i) ORF1. The ORF1 gene product showed high similarities to members of the superfamily of adenylate-forming enzymes, which includes all peptide synthases of bacterial and fungal origin and a series of adenylate-forming enzymes, such as luciferin 4-monooxygenase and acyl-CoA ligases (26). As shown in Fig. 3, the ORF1 protein has the minimal amino acid domains described for ATP-dependent adenylylating enzymes (26, 50). Domains A to I can be recognized within the ORF1 amino acid sequence, although domains A and H are not very well conserved. Figure 4 shows the alignment of these domains with those (ACV) of some representative peptide synthetases. The three modules of Aad-Cys-Val (ACV) synthesize activate the amino acids d-L-α-aminoadipic acid, L-cysteine, and L-valine, respectively, in the biosynthesis of β-lactams. Tyrocidin synthetase I activates the amino acid phenylalanine in tyrocidin biosynthesis. Finally, the Dae protein activates the amino acid D-alanine in the biosynthesis of D-alanyl esters of membrane-bound lipoteichoic acid.

As shown in Fig. 3 and 4, ORF1 (as DAE [12] and, probably, EntE [only a partial sequence is reported in reference 32]) shows the simplest structural organization thus far described for members of this family. Thus, ORF1 lacks domains involved in 4-phosphopantetheine attachment, those involved in condensation of the residues to form the skeleton of the final molecule, those involved in modification of the residues, and those presumptively involved in thioester bond hydrolysis (26, 50).

These findings allow us to assign to ORF1 a function of amino acid activation (β-lysine) through an ATP-dependent adenylation as in other peptide synthetases.

(ii) ORF2. The ORF2 product strongly resembles the gene products of mphA (38) and mphB (39) from E. coli (35.5% identity and 57.0% similarity and 29.5% identity and 49.0% similarity).
The corresponding genes, which encode type I (mphA) and type II (mphB) macrolide 2'-phosphotransferases, confer a high level of resistance to several macrolides in *E. coli*. This resistance mechanism is a consequence of a modification of the active macrolide by phosphorylation within the sugar moiety (28, 41). From the observed similarities, we can postulate that the ORF2 product might well be involved in phosphorylation of some intermediate substrate of the streptothricin biosynthetic pathway. Interestingly, the ORF2 protein includes several motifs that are highly conserved in some phosphorylating proteins (39, 47). Nevertheless, despite the observed similarities between the ORF2 and the type I and type II macrolide 2'-phosphotransferases, *S. rochei* F20 is highly sensitive to low levels (5 to 8 \(\mu\)g/ml in solid medium) of erythromycin or oleandomycin (unpublished observation), even under conditions in which streptothricin is produced.

(iii) ORF3. Database comparison of the ORF3 gene product showed a significant end-to-end resemblance with gene products whose functions are assigned to esterase activities from different origins (Table 2). It shows as much as 48.5% similarity to and 26.7% identity with the *Pseudomonas putida* esterase family related to TPES and as much as 48.3% similarity to and 31.2% identity with the related putative esterase group, whose members are, like ORF3, approximately 25 amino acid residues longer than the TPES family including the RdmC, DauP, and DnrP proteins. Together with ORF3, all of these proteins show as their most relevant features (i) a defined distribution of acidic residues (difficult to assign to a specific position because of the variable sizes of the esterases, although particularly located around positions 60, 80, 90, and 250 in the DauP-related proteins; (ii) a typically conserved hydrolase-active site, GxSxG, in which serine is the catalytic residue; and (iii) the strictly conserved histidine residue thought to be essential for function (located at position 276 for ORF3 as in DauP-related esterase proteins). The motif GxSxG, the conserved histidine residue, and the acidic residue located around position 250 constitute the so-called catalytic triad required for the charge-relay mechanism common to serine proteases, lipases, and cholinesterases (46). The secondary structure of the ORF3 gene product (Chou-Fasman and Garnier-Osguthorpe-Robson predictions) locates the GxSxG active site between the carboxy terminus of a \(\beta\)-strand and the amino terminus of an...
TABLE 2. Different proteins resembling the ORF3 gene product

<table>
<thead>
<tr>
<th>Protein</th>
<th>Origin</th>
<th>Reference</th>
<th>Presumptive function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPES</td>
<td>Pseudomonas putida</td>
<td>14</td>
<td>Atropinesterase</td>
</tr>
<tr>
<td>Est5</td>
<td>Pseudomonas putida</td>
<td>49</td>
<td>Carboxymethyl esterase</td>
</tr>
<tr>
<td>TODF</td>
<td>Pseudomonas putida</td>
<td>60</td>
<td>2-Hydroxy-6-oxo-2,4-heptadienoic acid hydrolase</td>
</tr>
<tr>
<td>XYL1F</td>
<td>Pseudomonas putida (pWW0)</td>
<td>18</td>
<td>2-Hydroxyxymonic semialdehyde hydrolase</td>
</tr>
<tr>
<td>DMDP</td>
<td>Pseudomonas putida (pVV1510)</td>
<td>40</td>
<td>2-Hydroxyxymonic semialdehyde hydrolase</td>
</tr>
<tr>
<td>RdMC</td>
<td>Streptomyces purpureascens</td>
<td>37a</td>
<td>Anthracycline biosynthesis</td>
</tr>
<tr>
<td>DauP</td>
<td>Streptomyces spp.</td>
<td>8</td>
<td>Daunomycin biosynthesis</td>
</tr>
<tr>
<td>DnRlP</td>
<td>Streptomyces peucetius</td>
<td>33a</td>
<td>10-Carbomethoxy-13-deoxycarminomycin esterase; involved in daunorubicin biosynthesis</td>
</tr>
</tbody>
</table>

α-helix, buried inside the protein, with a very low value of surface probability; this is similar, as revealed by high-resolution three-dimensional structure studies, to several hydrolases, such as two lipases and carboxypeptidase II (46).

The putative ORF3 active site GsSSG is also conserved in a number of thioesterases from different biochemical and phylogenetic origins, including fatty acid synthetase from vertebrates such as ducks and rats; polyketide synthase from bacteria such as erythromycin from Saccharopolyspora erythraea and candidin from Streptomyces griseus; and peptide antibiotics such as bialaphos from Streptomyces hygroscopicus, granicidin from Bacillus brevis, and penicillins from bacteria or fungi. An alignment of several putative thioesterase domains is shown in Fig. 5.

From all of this data, we postulate that ORF3 likely has a putative hydrolase activity. Whether it is related to hydrolysis of a thioester bond needs further investigation.

(iv) ORF4. Database searches with the translated product of ORF4 indicated no similarities to other known proteins and, therefore, no clue as to its possible function. However, strong similarities with a translated product of a DNA region from Streptomyces noursei which overlaps with the nourseothricin acetyltransferase gene (29) were shown.

(v) ORF5. A comparison of the ORF5 product sequence with those in databases revealed resemblance with several streptothricin acetyltransferases from different bacteria (Fig. 6). Thus, ORF5 shows a very high level of homology to those described from the gram-positive bacteria S. lavendulae (17) and S. noursei (29) and a significant but lower level of resemblance to those described from the gram-negative enterobacteriaceae Campylobacter coli (the so-called sat4 determinant [19]) and E. coli (the almost identical genes from transposons Tn7, Tn1825, and Tn1826 [the last two being the so-called sat1 and sat2 determinants] [52] and that from the IncQ R plasmid pIE639 [sat3 determinant] [51]).

In addition to a number of strictly conserved residues among these different streptothricin acetyltransferases, the alignments of these proteins reveal a core of conserved amino acids (residues 107 to 126) included in the previously described 66-amino-acid domain presumptively involved in the binding of acetyl-CoA or related cofactors (19, 54).

In order to detect a transacetylating activity in crude extracts of S. rochei F20, assays with culture broths from S. rochei F20 and from S. lavendulae (producer of streptothricin F) and the purified streptothricin mix from S. rochei F20 were made (see Materials and Methods). The results demonstrated the presence in both of these strains of an acetyl-CoA-dependent acetylation activity, which was sensitive to high temperature and able to inactivate streptothricin. Furthermore, subcloning of ORF5 in the E. coli expression vector pAZe3ss (59) yielded an E. coli strain resistant to streptothricin whose crude extract inactivated this antibiotic by acetylation, confirming without any doubt the involvement of this ORF in resistance to streptothricin via acetylation.

Insertional inactivation of ORF1, ORF2, and ORF3. Since the S. rochei F20 7.2-kb fragment containing ORF1 to ORF5 induces resistance but not antibiotic production in S. lividans, we had no biological clue as to the functions of ORF1 to ORF5. S. rochei F20 was shown not to be transformable with pU1101 (24), SCP2 (33), and pSG5 (37)-derivative replicons under standard conditions (15). However, during the course of this work, it was shown to be susceptible to infection by ΦC31 phage derivatives, and the implication of the ORFs in the biosynthesis of streptothricin was investigated by gene disruption. Thus, att3/C+ recombinant phages containing DNA fragments internal to ORF1, ORF2, and ORF3 were used (Fig. 2) on the phage vector KC515. These fragments were a 589-bp BsiI-BsiHI fragment (nucleotides 426 to 1015) for ORF1, a 1,066-bp XhoI/KpnI fragment (nucleotides 1804 to 2870) for ORF2, and a KpnI-PstI fragment (nucleotides 2875 to 3452) for ORF3. The recombinant phages were named φAB33, φAB30.1, and φAB31, respectively. All of these fragments were previously cloned onto an appropriate E. coli vector to make the ends compatible with the phage vector cloning sites.

The phages were isolated according to the method described by Hopwood et al. (15), and a suspension of confirmed recombinant phages was used to infect S. rochei F20. After sporulation, lysogens of the latter were detected either by replica plating or by harvesting and plating on thiostrepton-containing plates (selecting the φs marker of the phage vector). In all cases, the resulting chromosomal arrangements of lysogens were confirmed by Southern blot analysis.

Lysogenization with either φAB33 (ORF1) or φAB30.1 (ORF2) produced strains which were unable to produce any
detectable antibiotic activity in fermentation broth from liquid cultures. In contrast, lysogens obtained with AB31 (ORF3) yielded strains still producing detectable amounts of antibiotic, although at much lower levels than that in the wild-type strain. As a control for antibiotic production, lysogens of wild-type S. rochei F20 were constructed with recombinant phages carrying DNA from an unrelated region. The levels of antibiotic production by these lysogens were the same as those produced by the wild-type strain.

These results clearly show that ORF1, ORF2, and ORF3 (and perhaps ORF4) are indeed involved in the biosynthesis of streptothricin in our newly isolated S. rochei F20; thus, we suggest the following nomenclature for these characterized streptothricin biosynthetic genes: for ORF1, sttA; for ORF2, sttB; for ORF3, sttC; for ORF4, sttD; and for ORF5, sttR. Again, as in many other antibiotic biosynthesis clusters, the biosynthetic genes are linked together in a cluster with the resistance one.

**DISCUSSION**

Some biosynthesis genes for streptothricin production adjacent to the resistance gene have been isolated from S. rochei F20 and partially characterized. Although several similar resistance genes from different organisms had been previously described, to date there have been no data about the presence of linked related genes. Sequence analysis of the resistance gene from S. rochei F20 (ORF5) shows a very high level of similarity with previously reported streptothricin acetyltransferases from other Streptomyces spp. and, to a lower extent, with those from some enterobacteria. Crude extracts of S. rochei F20 and of E. coli expressing ORF5 inactivate, in the presence of acetyl-CoA, the antibiotic activity present in the fermentation broth from S. lavendulae, from S. rochei F20, and from the purified streptothricin mixture from S. rochei F20. Interestingly, the previously reported sequence of the streptothricin acetyltransferase gene from S. lavendulae (17) is very similar to the sequence reported here, although there are strong differences between the regions upstream of the genes; in our strain, unlike in the reported S. lavendulae gene, no ORF similar to ORF372 was found. This finding confirms that if the streptothricin acetyltransferase from S. lavendulae is linked to the stt biosynthetic genes, these might well be arranged differently from those reported here. In contrast, the finding of homology between ORF4, which overlaps with S. rochei F20 acetyltransferase, and a putative protein from S. noursei overlapping with the nourseothricin acetyltransferase gene suggests similar arrangements in both organisms, and, therefore, a possible linkage of the nourseothricin biosynthesis genes to the resistance gene in the producer organism.

Four ORFs linked to the resistance gene were found. By insertional inactivation, we can conclude that at least three of them (ORF1, -2, and -3) are involved in antibiotic biosynthesis. The deduced ORF1 protein shows a series of well-conserved domains identified in members belonging to the family of adenylate-forming enzymes. Within these domains, a series of
residues have been shown to be critical in some systems for function (for a review, see references 26 and 50), all of which are present in ORF1, which strongly supports the hypothesis that ORF1 belongs to this family of enzymes.

Domains D and E from gramicidin synthetase I and tyrocidine synthetase I, but not ACV synthetases and Dae protein, contain cysteine residues whose thiol groups seem to play an important role in enzymatic activity. In this sense, ORF1 would resemble ACVs and Dae more than gramicidin synthetase I and tyrocidine synthetase I.

The accepted mechanism for nonribosomal peptide synthesis is the so-called thioesterol mechanism, in which the amino acid or hydroxy acid is activated by adenylate and transferred to the -SH group of a 4'-phosphopantetheine anchored to the enzyme, from which it is transferred to another chemical group. ORF1 lacks the domain thought to be involved in 4'-phosphopantetheine attachment; thus, this theoretical mechanism does not seem to be attributable to ORF1. However, there are two situations in which this domain is not required: in the case of EntE, a noncovalent binding of the adlylated precursor to the enzyme has been reported (44), and in the case of d-alanine activation by Dae, the cofactor is anchored to a different peptide, which is highly homologous to ayl carrier proteins (ACPs) of fatty acids and polyketide biosynthetic systems (13). Meanwhile, d-alanine is covalently bound to the SH group of a cysteine residue of Dae. ORF1 contains two cysteines, which are located outside the putative functional domains and, therefore, could work as a receptor in the activation of the β-lactam moiety during streptothricin biosynthesis. Thus, the structural relationship between ORF1 and those concerned with peptide biosynthesis mediated by a nonribozymal mechanism, particularly with the Dae and EntE proteins, suggests the same general enzymatic mechanism.

The biochemical function of ORF2 is still unclear. Because of its resemblance to some phosphorylating proteins, we can speculate that this gene product could phosphorylate an intermediate of the streptothricin pathway, perhaps to prevent a hypothetical toxicity; the phosphorylated intermediate would be the substrate for another enzyme of the biosynthetic pathway. Similar suggestions about the involvement of a gene product in catalyzing a biosynthetic step and in resistance to active metabolites of the same process have been previously reported for other biosynthetic pathways, such as those for puromycin (55), bialaphos (36), and streptomycin (35). This hypothetical function needs some further biochemical characterization concerning the substrate specificity and its role in the biosynthetic pathway.

The resemblances shown by the ORF3 protein suggest an esterase activity. The exact function of thioesterases in nonribosomal peptide biosynthesis is unknown, but it is believed that they might hydrolyze the putative thioester bond to release the peptide product from the 4'-phosphopantethene enzyme (or cysteine residue in this case) and/or to catalyze the transfer of the growing peptide chain. In the case of streptothricin, the ORF1 gene product would be concerned with the activation and attachment of the β-lactam and recognition, perhaps with the collaboration of ORF2, of the substrate to be condensed with the activated amino acid. A final ORF3-mediated hydrolysis would release the product. Thus, in this biosynthetic cluster, the putative esterase activity would be located in an independent peptide, unlike in gramicidin synthetase II, ACVs, and other peptide synthases.

Mutagenesis by insertion inactivation of ORF1 and ORF2 abolished antibiotic production, and that for ORF3 reduced it significantly, suggesting their involvement in the biosynthetic pathway. The ORF3 internal fragment used for insertional inactivation included the putative thioesterase domain and three of the four aspartate residues believed to be important for activity. Lysogens would, therefore, contain two interrupted parts of ORF3, with one copy of the three aspartates and the putative thioesterase domain in each of them, which would therefore be duplicated. The residual activity of this structure might explain the low levels of antibiotic detected in these lysogens.

In summary, the peptide synthase for streptothricin biosynthesis could well involve a multienzymatic system, resembling to some extent the organization of type II polyketide synthases more than that of multifunctional proteins such as ACVs or type I polyketide synthases (for a review of polyketide synthases, see reference 16). Both processes, polyketide and nonribosomal peptide biosynthesis, lead to the synthesis of molecules by sequential steps which involve successive condensation of similar units. This makes both biochemical systems an example of convergent schemes for biosynthetic pathways developing similar architectural solutions for different biochemical processes.

ACKNOWLEDGMENTS

We thank D. A. Hopwood for critical reading of the manuscript. This work was supported by grants from the Spanish CICYT (BIO96-1168-C02-01 and BIO95-2072-E), Ministerio de Educación y Ciencia (Programa de Cooperación con Iberoamérica), and the Consejo Superior de Investigaciones Científicas/Agencia de Ciencia y Tecnología para el desarrollo de Cuba and by an institutional grant from Repsol Petroleo S.A. to Centro Nacional de Biotecnología.

REFERENCES

14. HESSING, F. on November 7, 2017 by guest http://jb.asm.org/ Downloaded from


