The Albonoursin Gene Cluster of S. noursei: Biosynthesis of Diketopiperazine Metabolites Independent of Nonribosomal Peptide Synthetases

Sylvie Lautru,1,2,4 Muriel Gondry,1 Roger Genet,1,3 and Jean-Luc Pernodet2,3
1CEA/Saclay
Département d’Ingénierie et d’Études des Protéines
F91191 Gif-sur-Yvette Cedex France
2Institut de Génétique et Microbiologie
CNRS UMR 8621, Bât. 400 Université Paris-Sud
F91405 Orsay Cedex France
3Université Paris-Sud
F91191 Gif-sur-Yvette Cedex France

Summary

Albonoursin [cyclo(L-Phe-L-Leu)], an antibacterial peptide produced by Streptomyces noursei, is one of the largest diketopiperazine (DKP) family. Formation of α,β unsaturations was previously shown to occur on cyclo(L-Phe-L-Leu), catalyzed by a pyroglutamate oxidase (CDO). We used CDO peptide sequence information to isolate a 3.8 kb S. noursei DNA fragment that directs albonoursin biosynthesis in Streptomyces lividans. This fragment encompasses four complete genes: albA and albB, necessary for CDO activity; albC, sufficient for cyclo precursor formation, although displaying no similarity to non ribosomal peptide synthetase (NRPS) genes; and albD, encoding a putative membrane protein. This first isolated DKP biosynthetic gene cluster should help to elucidate the mechanism of DKP formation, totally independent of NRPS, and to characterize novel DKP biosynthetic pathways that could be engineered to increase the molecular diversity of DKP derivatives.

Introduction

Diketopiperazine (DKP) derivatives (Figure 1A) constitute a family of secondary metabolites that are mainly produced by microorganisms. They were first discovered in 1880 and later studied by E. Fischer, who synthesized many of the simplest molecules, such as cyclic dipeptides [1]. These derivatives were long disregarded because many cyclic dipeptides formed from protein hydrolysates and found in culture media were considered to be by-products of protein degradation. During the last 30 years, however, more complex molecules bearing a piperazine-2,5-dione cycle have received an increasing amount of attention, as some of these compounds display diverse and interesting biological activities. These molecules include bicyclomycin (Biozam-ine), an antibacterial agent used as food additive to prevent diarrhea in calves and swine [2], and gliotoxin, the immunosuppressive properties of which are being evaluated for the selective ex vivo removal of immune cells responsible for tissue rejection [3]. Several compounds such as ambewelamides, verticillin, and phenyl-ahistin exhibit antifungal activities involving various mechanisms [4-6].

Although the number of newly isolated, naturally occurring DKPs has increased during the last few years, the biosynthetic pathways of these molecules remain largely unexplored. In bacteria and fungi, DKP derivatives generally seem to be produced by nonribosomal pathways, although their biosynthesis has never been shown to be directly catalyzed by NRPS megacomplexes. Studies of ergot alkaloid biosynthesis in the fungus Claviceps purpurea have shown that the α-lysergyl peptide synthetase, which is responsible for the formation of the α-lysergyl peptide-DKP, an intermediate in the biosynthesis of ergopeptines, is devoid of a C-terminal thioesterase domain. DKP cyclization is thus thought to occur spontaneously, leading to the release of the intermediate in solution [7].

Such spontaneous cyclization has also been observed in Bacillus brevis for the D-Phe-L-Pro dipeptide intermediate of gramicidin S biosynthesis and of tyrocidine A biosynthesis, due to the instability of the dipeptide thioester linkage during peptide elongation on peptide synthetase complexes [8]. In all of these cases, the formation of the DKP ring occurs in a molecule that is primarily activated via a covalent thioester linkage to the enzyme, and for which the cis conformation of the peptide bond, necessary for the cyclization reaction, is favored by the presence of a proline residue [8]. A similar mechanism may explain the formation of thaxtomins in Streptomyces disclobiaceus [9], where the N-methylation of the second residue also favors the cis conformation of the peptide bond [10].

The formation of naturally occurring DKPs has also been studied in humans and animals, in which the cyclic dipeptide cyclo(L-His-L-Pro) is derived from the thyrotropin-releasing hormone (TRH, pGlu-His-Pro). Here, also, the mechanism involves the nonenzymatic cyclization of the dipeptide L-His-L-Pro-NH₂, which results from the cleavage of the pyroglutamate residue from the TRH by a pyroglutamate amino peptidase [11].

Thus, in all of the known mechanisms of DKP formation, the primary structure of the precursor dipeptide, in particular the conformation of its peptide bond, appears to be a fundamental requirement. This raises questions concerning whether these mechanisms can be generalized for the synthesis of all piperazine-2,5-dione derivatives, and especially whether they can account for the biosynthesis of molecules that do not contain N-alkylated residues.

To investigate further the mechanisms of DKP formation, we studied the biosynthesis of albonoursin [cyclo(ΔPhe-ΔLeu)] (∆ indicating an α,α-β-dehydro residue) (Figure 1B), a secondary metabolite produced by Streptomyces noursei. We have shown in a previous study that the formation of α,β-unsaturated residues...
from the precursor cyclo(L-Phe-L-Leu), catalyzed by a novel enzyme named cyclic dipeptide oxidase (CDO), constitutes the final step of albonoursin biosynthesis [12]. The question thus arose: how is the cyclic dipeptide precursor synthesized?

Hypothesizing that the albonoursin biosynthetic genes would be clustered, as is the case for most secondary metabolite genes in *Streptomyces* species [13], we used CDO sequence information to isolate the corresponding gene cluster. We isolated from *S. noursei* a DNA fragment containing four genes that confer the ability to synthesize albonoursin to *Streptomyces lividans*. The partial characterization of the albonoursin gene cluster led us to identify the gene organization for CDO and provided evidence that specific enzymes, unrelated to NRPS, can catalyze the formation of DKP derivatives.

**Results**

**Isolation of a DNA Fragment Encoding CDO from *S. noursei***

Partial CDO amino acid sequences were needed to design oligonucleotides for the cloning of the corresponding gene by a PCR-based approach. As a posttranslational modification of the N-terminal residue prevented the direct determination of the native N-terminal sequence of CDO [12], we sequenced internal tryptic peptides. Following tryptic digestion, the resulting peptides were separated on a reverse phase column and further purified (if required) by size exclusion chromatography. Their masses were determined by MALDI-TOF mass spectrometry. Three of the 25 fractions analyzed were subjected to automatic sequencing by Edman degradation (see Experimental Procedures). The peptide sequences were used to design three forward (1f, 2f, and 3f) and three reverse (1r, 2r, and 3r) degenerate oligonucleotides, based on typical *Streptomyces* codon usage.

As the relative positions in the CDO sequence of the three peptides used to design the oligonucleotides were not known, six combinations of oligonucleotides had to be tested. Several PCR conditions were tried and numerous DNA fragments were amplified and sequenced, but none of them appeared to be a candidate for a CDO gene fragment: either the known peptide fragments were not translated from the same reading frame or amino acids downstream one of the known peptides were not as expected. A RT-PCR approach was therefore used with the hope that the complexity of the DNA mixture obtained after reverse transcription would be markedly reduced as compared to that of the whole genome. DNA from *S. noursei* was extracted from a 24 hr old culture grown in medium 5, conditions in which CDO activity is maximal. RT-PCR with the six combinations of oligonucleotides resulted in the amplification of a unique fragment of about 400 bp with oligonucleotides 3f and 2r. This fragment was cloned into the pGEM-T Easy vector and sequenced. Sequence analysis showed that the tryptic peptide sequences from which the oligonucleotides were derived were translated from the same reading frame. The third tryptic peptide sequence was not found in the protein sequence deduced from the 386 bp amplified DNA fragment, but the two amino acids following the “QAWSFMV” sequence (corresponding to oligonucleotide 3f) were V and R as expected. Furthermore, the presence of a lysine residue preceding the “NEVNY” sequence (corresponding to oligonucleotide 2r) was consistent with isolation of this peptide fragment from the tryptic digestion.

The amplified DNA fragment was used as a probe to screen a *S. noursei* genomic DNA library constructed in cosmid pWED1. *S. noursei* DNA was partially digested with BamHI, resulting in clones with insert sizes between 35 and 45 kb. About 2,000 clones were screened by colony hybridization and 12 were found to hybridize with the RT-PCR fragment. The corresponding cosmids were extracted and a Southern blot was performed on the BamHI-digested cosmids, using the RT-PCR fragment as a probe. This probe revealed a 3.8 kb BamHI fragment, common to all cosmids and also present in BamHI-digested *S. noursei* genomic DNA. This fragment was isolated from cosmid pSL117 and cloned into the pBC SK+ vector, yielding pSL122.

*E. coli* harboring pSL122 (denoted *E. coli[pSL122]*) was then directly tested on plates for CDO activity by detecting the accumulation of the yellow product, cyclo(Trp-Trp-Trp). *E. coli[pSL122]* displayed a clear yellow color after 16 hr of growth at 37°C (Figure 2). Disruption of cells followed by enzyme assay confirmed the presence of active CDO in the extract and showed that the BamHI fragment contained the genetic information for CDO.

**Sequence Analysis of the DNA Fragment Encompassing the CDO Coding Region**

The 3.8 kb BamHI fragment and one extremity of an overlapping NotI fragment subcloned from the cosmid pSL119 were sequenced. Two truncated open reading frames (ORFs) and four complete ORFs with typical *Streptomyces* codon usage [14] were identified (Figure 3). At the NotI extremity of the sequenced fragment, the product of the 5′-truncated ORF (orf1; 225 bp) showed a high level of similarity (78% identity and 87% similarity) to the C-terminal part of a probable tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase from *Streptomyces coelicolor* (SC05488) [15]. At the other extremity, the product of the 3′-truncated orf6 (1119 bp) strongly resembled the N-terminal part of a NADP-specific glutamate dehydrogenase from *S. coelicolor* (SCO4683) (78% identity and 86% similarity). Four complete ORFs (orf2, orf3, orf4, and orf5) were found between these two truncated ORFs and were transcribed in the same direction (Figure 3).
The first of these complete ORFs (orf2) included the RT-PCR amplified fragment, and the deduced amino acid sequence contained the sequences of the three known tryptic peptides. The product of orf2, therefore, corresponded to the 21 kDa protein isolated and purified from S. noursei [12]. Thus, this gene is clearly involved in albonoursin biosynthesis and was named albA. Sequence analysis indicated three possible start codons for albA, two GUG and one AUG, resulting, respectively, in 219, 204, or 196 amino acid proteins. However, as amino acid sequencing has shown that the N-terminal amino acid is blocked by a posttranslational modification, it was not possible to determine which of these codons corresponded to the true start codon. The most upstream possible start codon for albA is located 20 nt from one extremity of the BamHI fragment, which might therefore lack the promoter region for albA. The absence of a promoter region for albA in the BamHI fragment was confirmed in S. lividans (see below). Therefore, the albA promoter is probably located between the end of orf1 and the BamHI site. In E. coli [pSL122], the expression of CDO is most probably due to the presence of the lac promoter from the vector, located immediately upstream of albA. The sequence of albA revealed the presence of a UUA codon, which is rare in Streptomyces species and might be used for the translational regulation of gene expression [16]. Comparisons with databases showed that AlbA displays maximal similarity with a putative NADH oxidase from Archaeoglobus fulgidus (32% identity and 52% similarity), and searches for conserved domains indicated that it possesses a large nitroreductase domain (151 amino acids, pfam00881).

Immediately downstream of albA, Frame analysis clearly identified an ORF, orf3. The three possible start codons of orf3 were surprisingly all located in albA. Thus, the albA and orf3 coding regions overlap by 56, 41, or 23 bp, depending on the start codon used. BLAST and FASTA searches with the deduced product of orf3 did not reveal any significant similarity with any proteins in protein databases.

BLAST and FASTA searches with the deduced products of the last two ORFs did not help to determine their potential functions. orf4 starts most likely with an ATG and codes for a 239 amino acid protein, which is similar to two hypothetical proteins of unknown function, Rv2275 from Mycobacterium tuberculosis (34% identity and 53% similarity) and YvmC from Bacillus subtilis (29% identity and 46% similarity). The last complete ORF, orf5, encodes a 277 amino acid protein. This protein is probably a membrane protein as indicated by the analysis with TMHMM [17] and it resembles a probable membrane protein from S. coelicolor (SCO3332) (54% identity and 67% similarity).
orf3 Is Required for CDO Activity

Given that albA codes for the 21 kDa protein previously isolated from S. noursei and that E. coli[pSL122] expresses active CDO, we expected that E. coli harboring pSL127 (constructed by deleting all of the ORFs except albA from the BamHI fragment of pSL122) would express active CDO. However, no enzyme activity could be detected either on plates (Figure 2) or in cell extracts. Thus, we hypothesized that the expression of functional CDO requires the expression of another ORF in addition to albA, most likely orf3. To test this hypothesis, a plasmid (pSL145) containing albA and orf3 was constructed by deleting orf4 to orf6 from pSL122. E. coli[pSL145] did express active CDO (Figure 2), thus demonstrating that the product of orf3, now designated albB, is essential for CDO activity.

Production of Albonoursin in the Heterologous Host S. lividans

As the antibiotic biosynthetic genes are usually clustered in Streptomyces species and as we have demonstrated that AlbA and AlbB are part of the albonoursin biosynthetic pathway, the next question was are orf4 and orf5 also involved in the biosynthesis of albonoursin? The BamHI fragment was therefore cloned into the E. coli/Streptomyces shuttle vector, pUWL201, in the correct orientation so that all of the ORFs were under the control of the ermE' promoter, yielding pSL128. The BamHI fragment was also cloned in the opposite orientation, to give pSL129. S. lividans TK21 protoplasts were transformed with pSL128, pSL129, or pUWL201 (control) and the supernatants of 3-day-old cultures of the three transformed strains grown in medium 5 were analyzed by reverse phase HPLC. The S. lividans[pSL128] culture supernatant (Figure 4A) revealed two peaks with retention times of 40.4 min and 45.6 min, respectively. The major compound, exhibiting a UV spectra at (λmax) 318 nm and a molecular mass of 256.4 Da as determined by mass spectrometry, corresponds to albonoursin [18]. The second compound, identified as cyclo(ΔPhe-ΔPhe) from its UV-visible spectrum (λmax 338 nm) and its molecular mass (290.3 Da), has previously been shown to be produced concomitantly with albonoursin by S. noursei [19]. Both of these compounds were absent in the control culture (Figure 4B). Furthermore, S. lividans[pSL129] did not produce any albonoursin (Figure 4C), which confirms that the BamHI fragment does not contain the promoter for albA.

Albonoursin was also detected in the culture supernatant of S. lividans[pSL144] (data not shown). In pSL144, the four genes albA, albB, orf4 and orf5 are cloned into pUWL201, under the control of the ermE' promoter, but orf6 is deleted. This confirms that the truncated orf6 plays no role in albonoursin biosynthesis.

The production of albonoursin by S. lividans[pSL128] does not prove, however, that orf4 and orf5 are involved in albonoursin production, as S. lividans may produce the albonoursin precursor, cyclo(L-Phe-L-Leu), which could thereafter be converted into albonoursin by CDO. To test this hypothesis, albA and albB were cloned into pUWL201, yielding pSL142, and the culture supernatant of S. lividans[pSL142] was analyzed by HPLC (data not shown). No albonoursin was found in the culture supernatant, although enzyme assays showed that the cell extract contained active CDO. Thus, cyclo(L-Phe-L-Leu) was not synthesized by S. lividans TK21. This strongly suggests that at least one of orf4 and orf5 is involved in the biosynthesis of albonoursin.

orf4 Is Sufficient for the Biosynthesis of the Albonoursin Precursor, Cyclo(L-Phe-L-Leu)

To confirm the above results, orf4 and orf5 were cloned into pUWL201, yielding pSL167, which was introduced into S. lividans TK21. The supernatant of a S. lividans[pSL167] culture was then analyzed to determine whether cyclo(L-Phe-L-Leu) was produced. Direct detection of this molecule in the culture medium is difficult because of its weak absorption coefficient (εmax = 200 M−1cm−1 at its maximal absorption wavelength, 254 nm). Thus, we converted any cyclo(L-Phe-L-Leu) potentially present in the culture supernatant into albonoursin, which is
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Figure 5. Amino Acid Sequence Alignment of AlbA with FMN binding Signatures of Other “Nitroreductase Domain”-Containing Enzymes

The amino acid sequence of AlbA was aligned manually with structure-based alignments previously proposed for NTR (FMN-dependent nitroreductase from *E. coli* B), FRS 1 (NAD:FMN oxidoreductase enzyme from *Vibrio fischeri*), NOX (NADH oxidase from *Thermus thermophilus*), and FRP (NADPH-dependent flavin reductase from *Vibrio harveyi*) [21], and for NfsA (major oxygen-insensitive NADPH-dependent nitroreductase from *E. coli*) and FRP [23]. Residues that are identical in three or more of the five nitroreductase domains are highlighted in yellow, as are the conserved residues in the AlbA alignment. Residues that are shared by AlbA and at least two reference protein sequences are highlighted in blue. Gaps are shown by the symbol ‘-’; amino acid residues involved in FMN binding for the five proteins of known structure are shown in red, and those which are conserved in AlbA are boxed.

m much more easily detected as it has a $\varepsilon_{\text{mol}} = 25,400$ M$^{-1}$cm$^{-1}$ at 318 nm [18]. This was done by incubating the culture supernatant of *S. lividans* [pSL167] at 30°C overnight in the presence of $4.1 \times 10^{-3}$ units of purified CDO. HPLC analysis revealed the presence of albonoursin in this fraction, identified by UV-visible spectroscopy and mass spectrometry. However, no albonoursin was present in the *S. lividans* [pSL167] supernatant that had not been incubated with CDO or in the supernatant of *S. lividans* TK21 that had been incubated with CDO (data not shown, see below). This clearly shows that at least one of the two ORFs, orf4 or orf5, is involved in albonoursin biosynthesis.

Each ORF was then separately cloned into pUWL201 and the resulting plasmids (pSL168 for orf4 and pSL159 for orf5) were introduced into *S. lividans* TK21. HPLC analysis of *S. lividans* [pSL168] culture supernatants demonstrated the presence of albonoursin only when incubated with CDO (Figures 4D and 4E). No albonoursin could be detected in the *S. lividans* [pSL159] culture supernatant with or without CDO (Figures 4F and 4G).

Altogether, these results show that the product of orf5 does not directly participate in the biosynthesis of the cyclo(L-Phe-L-Leu) and that the presence of orf4, from now on designated albC, is sufficient for the production of this molecule by *S. lividans*.

**Discussion**

The aim of this study was to isolate and to characterize the biosynthetic pathway of albonoursin, a bis-dehydrocyclic dipeptide produced by *S. noursei* that displays antibacterial and antitumor activities [20]. We first showed that a 3.3 kb BamHI fragment isolated from *S. noursei* DNA contains all of the information needed for the production of albonoursin by *S. lividans*, a bacterium that does not naturally synthesize this compound. This confirms that the albonoursin biosynthesis genes are clustered and indicates that all of the genes required for albonoursin biosynthesis are included within the BamHI fragment. Sequence analysis revealed the presence of four complete ORFs in this locus (Figure 3). These ORFs are surrounded by gene fragments coding for proteins not involved in the albonoursin pathway: a probable tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase, upstream, and a probable NADP-specific glutamate dehydrogenase, downstream. This allowed us to delimit the albonoursin gene cluster to the four complete ORFs, transcribed in the same direction, included in the BamHI fragment.

The first of the four ORFs of the albonoursin pathway, albA, encodes a 21 kDa protein (AlbA) that contains all of the tryptic sequences, as determined by studying a purified enzymatic fraction of *S. noursei* exhibiting CDO activity [12]. Sequence analysis indicated that AlbA displays a fairly high degree of sequence similarity with the pfm00881 domain, also called “nitroreductase domain,” found in nitroreductases [21] and in some flavin reductases [22, 23] and NADH oxidases [24]. Like all of these enzymes, CDO catalyzes an oxidoreduction reaction requiring the presence of a flavin cofactor. However, the electron donor for the enzymes possessing a nitroreductase domain identified up to now is always NAD(P)H. This is not the case for CDO, which catalyzes the oxidation of cyclic dipeptides. Furthermore, CDO contains a covalently bound flavin cofactor [12], whereas FMN is tightly but not covalently bound in the other nitroreductase domain-containing enzymes.

The alignment of the sequence of AlbA with the sequences of the enzymes for which the structures have been solved and in which the residues involved in FMN binding have been determined showed that most of FMN binding residues are conserved in AlbA (Figure 5). Our results demonstrate that *S. lividans* and *E. coli* containing only albA do not produce an active CDO and that the immediately downstream gene, albB, is also required to obtain an active enzyme. A band with a relative molecular mass (Mr) of 11,000 that could corre-
The third and last ORF that is directly involved in albonoursin biosynthesis is \textit{albc}, encoding a 239 amino acid protein of 26.8 kDa (AlbC) that does not resemble any functionally characterized proteins. Nevertheless, our experiments allowed us to assign a role to this protein in albonoursin biosynthesis. Indeed, the expression of \textit{albc} by \textit{S. lividans} TK21 or \textit{E. coli} (data not shown) led to the production of cyclo(\textit{l}-\textit{Phe}-\textit{l}-\textit{Leu}), an intermediate in albonoursin biosynthesis and a substrate of CDO. These two strains also produce a second cyclic dipeptide cyclo(\textit{l}-\textit{Phe}-\textit{l}-\textit{Phe}), which, when converted by CDO, yields cyclo(\textit{\Delta Phe}-\textit{\Delta Phe}). This second dehydro-DKP metabolite has been isolated together with albonoursin from \textit{S. noursei} [19], although the relative amounts of each of these two metabolites greatly vary with culture conditions and the duration of cultivation.

These results led to the conclusion that AlbC catalyzes the condensation of two amino acids to form cyclic dipeptides [cyclo(\textit{l}-\textit{Phe}-\textit{l}-\textit{Leu}) or cyclo(\textit{l}-\textit{Phe}-\textit{l}-\textit{Phe})] by an unknown mechanism. In all of the cases described to date, the formation of a peptide bond involves an ATP-dependent mechanism in which amino acids are activated via an amino acyl adenylate or via the formation of an acyl phosphate intermediate. In the first case, enzymes catalyzing the transient formation of amino acyl adenylates share conserved nucleotide binding domains that are easily identified by their sequence signature (“AMP binding” domain present in NRPS, PROSITE sequence PS00455). In the second case, enzymes catalyzing the formation of a peptide bond via an acyl phosphate intermediate, such as glutathione synthetase, D-Ala-D-Ala ligase, or MurD, present only a very low level of sequence similarity. Several of these enzymes present an ATP binding motif called “ATP grasp” [25]. This motif is characterized by a common structural organization of the amino acids involved in binding of the substrates and nucleotide, although they do not share any consensus patterns for the ATP binding site. The sequence comparisons and the crystallographic data analysis clearly showed that enzymes that use ATP-dependent mechanisms display great structural diversity in their nucleotide binding domains. Moreover, Keefe and Szostak [26] were able to select, from a random sequence peptide library, four new ATP binding proteins that are unrelated to each other or to any proteins currently found in the databases. Thus, the way in which a protein can accommodate ATP is not restricted to the situations that have been described in natural proteins.

Comparison of the AlbC sequence with databases revealed two proteins of unknown function with moderate similarity (Rv2275 from \textit{Mycobacterium tuberculosis} [34% identity, 53% similarity] and YvmC from \textit{Bacillus subtilis} [29% identity, 46% similarity]). Although these proteins share a conserved region (amino acids 35–45 in AlbC; data not shown) that is rich in glycine and serine, they do not present any of the typical signatures of nucleotide binding domains (e.g., “AMP binding” domain, “P loop” PROSITE sequence PS00017, “putative ATP binding domain of some kinases” PROSITE sequence PS00627). Attempts to predict AlbC secondary structure elements and to compare them with structure databases did not reveal any significant structural similarity.

Thus, AlbC is the first example of an enzyme that is directly involved in the synthesis of DKP derivatives, except for NRPSs for which DKPs are produced as by-products, via an uncatalyzed cyclization reaction [7, 8]. Further studies are needed to characterize the mechanism of the reaction(s) catalyzed by AlbC. It would be particularly interesting to determine whether the cyclization reaction is directly catalyzed by AlbC, as the structures of the potential precursors (\textit{l}-\textit{Phe}-\textit{l}-\textit{Leu} or \textit{l}-\textit{Leu}-\textit{l}-\textit{Phe}) do not favor a \textit{cis} conformation of the peptide bond as is generally the case for the formation of DKPs containing prolyl or \textit{N}-methyl-amino acyl residues. Nevertheless, our experiments unambiguously showed that no enzymes other than AlbC are required for the synthesis of DKPs.
Table 1. Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Properties</th>
<th>Source/Reference</th>
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<td>Bacterial Strains</td>
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<tr>
<td>E. coli DH5α</td>
<td>General cloning host</td>
<td>Invitrogen</td>
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<td>E. coli SURE</td>
<td>Host strain for the cosmid library</td>
<td>Stratagene</td>
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<td>S. lividans TK21</td>
<td>Streptomyces host strain for cloned (\text{alb}) genes</td>
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<td>Wild-type strain producing albonoursin</td>
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<td>Cloning vector for PCR products, Amp(^R)</td>
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<td>Cosmid vector derived from pWE15, Amp(^R)</td>
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<td>pBC SK(^H)</td>
<td>Cloning vector, Cm(^R)</td>
<td>Stratagene</td>
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<td>E. coli/Streptomyces shuttle vector, contains the (\text{ErnE}) promoter for expression of cloned genes in Streptomyces. Amp(^R), Thio(^R)</td>
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<tr>
<td>pSL166</td>
<td>PCR product from (\text{albC} + \text{albD}) amplification cloned into pGEM-T easy</td>
<td>This work</td>
</tr>
<tr>
<td>pSL167</td>
<td>PstI/Klenow/BamHI fragment from pSL166 cloned into pUWL201</td>
<td>This work</td>
</tr>
<tr>
<td>pSL168</td>
<td>PstI/Klenow/BamHI fragment from pSL165 cloned into pUWL201</td>
<td>This work</td>
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</tbody>
</table>

The synthesis of the cyclic dipeptides cyclo(L-Phe-L-Leu) and cyclo(L-Phe-L-Phe) in S. lividans and E. coli.

Taken together, these results allowed us to propose a biosynthetic pathway for albonoursin, starting from L-Leucine and L-Phenylalanine and comprising at least two steps: the formation of the cyclic dipeptide and the subsequent successive \(\alpha,\beta\)-dehydrogenation of the two amino acid side chains (Figure 6). Finally, the role of \(\text{orf5}\), the fourth ORF of the albonoursin gene cluster, has to be considered.

Structure prediction algorithms suggested that the \(\text{orf5}\) product (named \(\text{AlbD}\)) contains a large membrane spanning domain, and sequence analyses showed that it displays a high level of sequence similarity with a probable membrane protein of unknown function in S. coelicolor. Therefore, as \(\text{AlbD}\) is not strictly necessary for albonoursin biosynthesis, we propose that it is involved in the transport of albonoursin across membranes or serves as a target for albonoursin acting in the same way as other small diffusible molecules in intercellular communication, in a phenomenon known as "quorum sensing." DKPs and \(\alpha,\beta\)-dehydro-DKPs display a remarkable spectrum of biological and pharmacological activities. These include their role in bacterial...
signaling systems, as recently established in Pseudomonas aeruginosa and other gram-negative bacteria [27, 28], which might suggest a comparable role for albonoursin in Streptomyces noursei.

Significance

We have identified in Streptomyces noursei the complete biosynthetic gene cluster for the production of albonoursin, a representative DKP-derived metabolite characterized by a typical piperezine-2,5-dione nucleus substituted by two α,β-dehydroamino acyl side chains. A gene cluster of about 2.7 kb constituted of four ORFs named albA, albB, albC, and albD has been isolated. When transferred into S. lividans, this small cluster is sufficient to confer the capacity to produce albonoursin. Sequence analysis allowed us to show that AlbA is the 21 kDa subunit of CDO, an enzyme we have previously shown to catalyze the α,β-dehydrogenation of the amino acyl side chains of the cyclic dipeptide intermediate cyclo((L-Phe-L-Leu)). Here we show that a second protein, AlbB, is essential for CDO activity. The third component of the biosynthetic pathway, AlbC, is of particular interest as it is directly involved in the formation of the DKP derivatives, cyclo((L-Phe-L-Leu)) and cyclo((L-Phe-L-Phe)), both of which are produced, although in variable proportions, by S. noursei. Thus, we have demonstrated that these two metabolites are derived from a unique biosynthetic pathway. AlbC, which has no similarities with NRPS multienzymes, is probably the first example of an enzyme that is directly involved in the cyclization of the DKP motif. Understanding this peculiar biosynthetic mechanism will open the way to the elucidation of new biosynthetic pathways of biologically interesting diketopiperazines which constitute a growing family of secondary metabolites exhibiting a wide variety of biological and pharmacological activities.

Experimental Procedures

Bacterial Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. E. coli strains were grown at 37°C in LB medium. Streptomyces strains were cultivated at 30°C either in ATCC medium 5 or in TS/medium (Tryptic Soy Broth, Difco).

Other Materials

Antibiotics (ampicillin [Amp], chloramphenicol [Cm], thiostrepton [Thio], apramycin [Apr]) were purchased from Appligene or Sigma-Aldrich. Restriction enzymes (BamHI, Apal, EcoRI, NdeI, HindIII, Asp718I, XbaI, PstI, and NotI) and DNaseI were purchased from the manufacturers’ instructions. Cyclo((L-Trp-L-Trp)) was purchased from Bachem. All other chemicals, including HPLC pure trypsin and protein standards for SDS-polyacrylamide gel electrophoresis, were from Sigma-Aldrich. CDO was purified from S. noursei as previously described [12] and stored at −80°C.

In Gel Tryptic Digestion and Peptide Sequencing

A pure CDO fraction was subjected to electrophoresis in a 15% SDS-PAGE gel and stained with Coomassie brilliant blue R. The 21 kDa band, corresponding to the CDO subunit, was excised together with a blank piece of gel (control). The two pieces of gel were digested in 1 ml of 50 mM Tris-HCl (pH 8.0) using HPLC pure trypsin in a 1:50 enzyme/substrate ratio, for 20 hr at 37°C. The peptides were then separated on a reverse phase column (μRPC C18, SC21/10, Pharmacia) using a linear acetonitrile gradient (0% to 76% in 62 min). The purity of the collected fractions was assessed by MALDI-TOF mass spectrometry. The peptides were further purified by size exclusion chromatography on a superdex peptide PC32/30 column (Pharmacia) equilibrated with a buffer containing 30% acetonitrile and 0.1% trifluoro acid. Three peptides were sequenced by Edman degradation (sequencer model 477A, Applied Biosystems): peptide 1, EPVDALLELEMEALAPTP; peptide 2, NEVNYEWGN; peptide 3, QAXSMVFR (X: undetermined amino acid). Comparison of the calculated and experimental masses of peptide 3 showed that the undetermined amino acid was tryptophan. The underlined and bold sequences were used to design the forward and reverse oligonucleotides (see below).

Amplification of a Gene Fragment by RT-PCR

DNA from Streptomyces noursei was extracted as described by Kieser et al. [13], with an additional treatment with DNaseI to ensure complete elimination of DNA. Degenerate forward and reverse oligonucleotides were deduced from peptide sequences given above (oligo 1f, GACCGTSAGCCAGGCAC; oligo 1r, CGCTCGTSCAGGCYT; oligo 2f, AACGARGTSTGSAACTCAGA; oligo 2r, TCCTGTGGTACACGCYT; oligo 3f, CAGCSTGGWSSCTTATCGT; oligo 3r, ACCATGAASSWCCASGCCTG [R = A or G; S = C or G; Y = C or T and W = A or T]) and synthesized (Sigma Genosys). RT-PCR was carried out using the Titan One Tube RT-PCR kit (Roche) according to the instructions of the manufacturer with 1 μg of total RNA for each reaction. Reverse transcription was performed at 50°C for 30 min and PCR conditions were as follows: initial denaturation at 97°C for 4 min followed by 45 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 68°C, and a final polymerization reaction at 68°C for 10 min. The PCR products were analyzed on a 1.5% agarose gel, purified (DNA and Gel Band purification kit, Pharmacia), cloned into the pGEM-T easy vector, and sequenced.

DNA Manipulations and Cloning Procedures

DNA extraction and manipulation, E. coli transformations, and Streptomyces lividans TK21 protoplast preparations and transformations were performed according to standard procedures [13, 33]. pSL117 and pSL119 are cosmids from the S. noursei genomic library containing the ORFs coding for CDO. pSL122 contains a BamHI fragment carrying all of the alb genes. pSL127 and pSL145 were constructed by digesting pSL122 with Apal or EcoRI, respectively, and religation. pSL142 and pSL144 were constructed in two steps: pSL122 was digested with EcoRI and treated with Klenow and digested with HindIII-Klenow-digested pSL138, or digested with NdeI and treated with Klenow and ligated together with the HindIII-Klenow-digested (lac cassette to yield pSL138 and pSL140, respectively. These plasmids were then digested with Asp718I, Klenow, and then BamHI and the resulting fragments containing orf2, orf3, and the lac cassette, or orf2 to orf5 and the lac cassette were cloned into pUWL201 that had been digested with XbaI-Klenow and then BamHI.

The primers SYLV24 (5'-CGGCTGACAGGAAAGAGGACCGACA TATGCTTGAAGCATTGGTCCTCC-3', PstI site underlined) and SYLV22 (5'-CGGCTCCGTGATCCAGCTTTCAAGGGCGGCGGGCTCTG-3', BamHI site underlined) were used to PCR amplify orf4. The primers SYLV19 (5'-GACGCGATCTTCGAATGTCGACGACGGTCGTCGCTCT-3', PstI site underlined) and SYLV18 (5'-CGTACAGGGAT CCAGGATCTTTAATGTCGACGACGGTCGTCGCTCT-3', BamHI site underlined) were used to PCR amplify orf5. The primers SYLV24 and SYLV18 were used to PCR amplify (orf4 to orf5). As orf3 and orf4 are only separated by 37 bp, a synthetic ribosome binding site was included in SYLV24 to ensure a good translation of orf4. The PCR-amplified fragments were cloned into the pGEM-T easy vector (Promega) to yield pSL165, pSL167, and pSL166, respectively. PstI-BamHI fragments from these plasmids were then cloned into pUWL201 that had been digested with PstI-BamHI to yield pSL168, pSL159, and pSL167, respectively.

Construction and Screening of the DNA Library

Genomic DNA extracted from S. noursei was partially digested with BamHI at about 35–45% to yield DNA fragments of 2–5 kb. These fragments were ligated with the pWE120 cosmid vector that had first been digested by BamHI and dephosphorylated. The ligated DNA was
packaged in vitro into lambda phages with the Packagene Lambda DNA packaging system (Promega), and E. coli (SURE) were infected by phage particles. The RT-PCR amplified DNA fragment was labeled by random priming with [α-32P]dCTP using the T7 Quick Prime kit (Pharmacia) and used as a probe to screen the library.

DNA Sequencing and Sequence Analysis
DNA sequencing was carried out on an ABI PRISM Genetic analyzer (Perkin Elmer) using the DYEnamic ET terminator cycles kit (Pharmacia) or by the Genome Express company. The Frame analysis [14] was used to identify ORFs. Sequences were compared with databases with the BLAST and FASTA programs [34, 35].

Detection of CDO Activity
A colorimetric test was developed to detect CDO activity within bacteria grown on plates. We looked for a substrate of CDO that yielded a colored and if possible insoluble compound once converted. The colorless cyclo([-Trp-]-Trp) fulfilled those two conditions, as it can be efficiently converted by CDO into cyclo([-L-Trp-]-Trp) (λmax = 367 nm; λmax = 450 nm), a barely water-soluble yellow compound [12]. The test was developed using S. noursei grown on plates containing between 0 and 1 mM cyclo([-L-Trp-]-L-Trp). After 7 days of culture at 30°C, bacteria grown on media containing cyclo([-L-Trp-]-L-Trp) at concentrations as low as 0.5 mM clearly exhibited a yellow coloration, whereas those grown on the control medium did not. Therefore, the standard test conditions for the detection of CDO activity in Streptomyces or E. coli were defined by the addition of 0.5 mM cyclo([-L-Trp-]-L-Trp) to the growth medium.

Detection of CDO activity in solution was performed in the standard conditions [12] by following the conversion of the model substrate cyclo([-Phe-]-L-His) into cyclo([-Phe-]-L-His) at 297 nm, in 100 mM Tris-HCl buffer (pH 8), at 30°C.

HPLC Analysis of Metabolites in the Culture Filtrates
Various metabolites, including albonoursin, were detected in culture supernatants by HPLC analysis. Culture supernatants (500 μl) were filtered through ultrafilter-TC (10 kDa cut-off, Millipore). Filtrates were eluted by reverse phase chromatography on a C18 column (4.6 × 250 mm, Vydac) using a 0%-45% linear gradient of acetonitrile in 0.1% trifluoroacetic acid for 45 min (flow rate 1 ml/min). The elution was monitored by a multi-wavelength detector between 200 and 600 nm. For detection of cyclosporin peptides, culture filtrates were incubated overnight at 30°C with 4.1 × 10^4 units of purified CDO. They were then analyzed by HPLC together with control culture filtrates that had not been incubated with CDO. The masses of the metabolites were determined by electrospray mass spectrometry by Atheris Laboratories (Switzerland).

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Accession Numbers

The complete DNA sequence of the albonoursin biosynthetic gene cluster has been deposited in the Genbank database with the accession number AY129235.