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Molecular Cloning of the Actinomycin Synthetase Gene Cluster from *Streptomyces chrysomallus* and Functional Heterologous Expression of the Gene Encoding Actinomycin Synthetase II

FLORIAN SCHAUWECKER,[1] FRANK PFENNIG,[1] WERNER SCHRÖDER,[2] AND ULLRICH KELLER[1]*

Max-Volmer-Institut, Fachgebiet Biochemie und Molekulare Biologie, Technische Universität Berlin, D-10587 Berlin-Charlottenburg,[1] and Institut für Biochemie, Sfb 344, Freie Universität Berlin, 14195 Berlin-Dahlem,[2] Germany

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The actinomycin synthetases ACMS I, II, and III catalyze the assembly of the acyl peptide lactone precursor of actinomycin by a nonribosomal mechanism. We have cloned the genes of ACMS I (*acmA*) and ACMS II (*acmB*) by hybridization screening of a cosmid library of *Streptomyces chrysomallus* DNA with synthetic oligonucleotides derived from peptide sequences of the two enzymes. Their genes were found to be closely linked and are arranged in opposite orientations. Hybridization mapping and partial sequence analyses indicate that the gene of an additional peptide synthetase, most likely the gene of ACMS III (*acmC*), is located immediately downstream of *acmB* in the same orientation. The protein sequence of ACMS II, deduced from *acmB*, shows that the enzyme contains two amino acid activation domains, which are characteristic of peptide synthetases, and an additional epimerization domain. Heterologous expression of *acmB* from the *mel* promoter of plasmid PIJ702 in *Streptomyces lividans* yielded a functional 280-kDa peptide synthetase which activates threonine and valine as enzyme-bound thioesters. It also catalyzes the dipeptide formation of threonyl–L-valine, which is epimerized to threonyl–D-valine. Both of these dipeptides are enzyme bound as thioesters. This catalytic activity is identical to the in vitro activity of ACMS II from *S. chrysomallus*.

The actinomycins are a class of chromopeptide lactones produced by various *Streptomyces* strains. They contain two pentapeptide lactone rings attached to chromophoric 4,6-dimethylphenoxazinone-1,9-dicarboxylic acid (actinocin) in an amide-like fashion. Actinocin is formally derived from the compound 4-methyl-3-hydroxyanthranilic acid (4-MHA), but actually the bicyclic actinomycins arise from the oxidative condensation of two preformed monocyclic 4-MHA pentapeptide lactones (12). Previous investigations have revealed that the formation of the 4-MHA pentapeptide lactones is catalyzed by three actinomycin synthetases (ACMS I, II, and III) (13, 15). ACMS I (45 kDa) is a 4-MHA–AMP ligase which activates 4-MHA as adenylate. The five amino acids of the pentapeptide lactone ring of actinomycin (NH_2-cyclo[Thr–D-Val–Pro–*N*-methyl-Gly–*N*-methyl-Val] for actinomycin D) are assembled by ACMS II (280 kDa) and ACMS III (480 kDa) which from their properties belong to the class of peptide synthetases (13, 26, 27). ACMS II catalyzes the activation of threonine and valine. In the presence of ACMS I, which supplies 4-MHA–adenylate, 4-MHA–threonine and 4-MHA–threonyl–D-valine (via 4-MHA–threonyl–L-valine) are formed on the surface of ACMS II. In the absence of 4-MHA or ACMS I, purified ACMS II can synthesize both threonyl–L-valine and threonyl–D-valine, though to a lesser extent than the corresponding 4-MHA dipeptides can. The epimerization of valine is catalyzed by ACMS II at the acyl-dipeptide stage. An analysis of ACMS III suggests that it elongates the 4-MHA–Thr–D-Val dipeptide by successive incorporation of proline, *N*-methylglycine (sarcosine), and *N*-methyl-L-valine into the growing peptide chain (13). N-methylation is an additional feature of ACMS III. A total cell-free system for 4-MHA pentapeptide lactone synthesis is not available yet. Thus, it is not known how 4-MHA dipeptide transfer from ACMS II to ACMS III is accomplished, nor is the mechanism of lactone formation and release from the 4-MHA pentapeptide known.

The available data indicate that ACMS II and ACMS III contain two- and three-amino-acid activation domains, respectively. It is known that activation domains of peptide synthetases are highly conserved in their sequences and are composed of a segment for amino acid adenylation and a segment for binding the activated amino acid as a thioester (17, 24, 25, 32). Thioester formation occurs via the thiol group of 4′-phosphopantetheine, which is a covalently bound cofactor of the activation domain. ACMS II and III both contain 4′-phosphopantetheine. In contrast, ACMS I has no 4′-phosphopantetheine cofactor, consistent with the finding that it does not form a thioester with 4-MHA. Data from previous work pointed instead to the formation of a 4-MHA thioester with ACMS II (26). In order to investigate the modular structure of the ACMSs and the reaction mechanisms in more detail, we set out to clone the ACMS genes from *Streptomyces chrysomallus* with oligonucleotide probes derived from partial sequences of ACMS I and II. We show that the genes of ACMS I and II and of a third peptide synthetase, most probably the gene of ACMS III (*acmA*, *acmB*, and *acmC*, respectively) are closely linked, forming a gene cluster. A total sequence determination of *acmB* and the characterization of the heterologously expressed functional active gene product confirm the significance of this peptide synthetase gene cluster.

MATERIALS AND METHODS

Strains and growth of organisms. *Streptomyces lividans* 1326 (John Innes Collection) was maintained at 30°C on R5 plates (10). Submerged growth took place for 2 to 3 days in 100 ml of YEME liquid medium (10) in 300-ml flasks equipped with steel springs and shaken at 200 rpm. The transformation of *S. lividans* was performed as described by Hopwood et al. (10). For heterologous expression of *acmB* in *S. lividans*, transformants harboring plasmid pACM5 were

* Corresponding author. Mailing address: Max-Volmer-Institut, Fachgebiet Biochemie und Molekulare Biologie, Technische Universität Berlin, Franklinstrasse 29, D-10587 Berlin-Charlottenburg, Germany. Phone and fax: 49 30 314 73522. E-mail: Ullrich@chem.TU-Berlin.de.

grown for 2 days in 100 ml of YEME–5 μg of thiostrepton per ml; 5 ml of glucose (20% [wt/vol]) was added, and growth was continued for 1 day. *Escherichia coli* strains were DH5α and DH1 (7).

Protein purification from *S. lividans*. Ten grams of mycelium (wet weight; harvested by suction filtration) was suspended in 100 ml of ice-cold buffer AS (10% glycerol [wt/vol], 200 mM Tris-HCl [pH 8], 30 mM $MgCl_2$, 10 mM 1,4 dithiothreitol, 1 mM benzamidine, 1 mM EDTA, 5 mM phenylmethylsulfonyl fluoride [PMSF]). After passage of the suspension through a French press at 10,000 lb/in^2, 1 mg of DNase I (Sigma; 90% protein = 440 Kunitz U) was added and the homogenate was stirred for 40 min on ice. Buffer AZ (95% glycerol [wt/vol], 850 mM NaCl) was added to give a final concentration of 300 mM NaCl, and after centrifugation (20 min, 15,000 × *g*, 4°C) 0.03 volume of neutralized polymin P (BASF; 10% [wt/vol]) was added to the supernatant. After standing on ice for 30 min, the precipitate was removed by centrifugation (25 min, 15,000 × *g*, 4°C). Ammonium sulfate (saturated solution at 4°C) was added to give 60% saturation, and after incubation for 5 h on ice the protein precipitate was collected by centrifugation (30 min, 15,000 × *g*, 4°C). Proteins were resuspended in buffer B (15% glycerol [wt/vol], 100 mM Tris-HCl [pH 8], 4 mM dithiothreitol, 1 mM benzamidine, 1 mM EDTA, 1 mM PMSF). Protein portions of 100 mg were purified by gel filtration on an Ultrogel-AcA-34 column at 4°C (Biosepra; range, 20 to 350 kDa; 2 by 48 cm) with buffer B and a fraction volume of 4.2 ml. For fast protein liquid chromatography (FPLC) purification, Ultrogel-AcA-34 fractions showing thioester formation activity with [^{14}C]Val and [^{14}C]Thr (e.g., fractions 14 to 19 in Fig. 4) were applied to a Resource-Q column (Pharmacia; polystyrene-divinyl benzene-based anion exchanger) at room temperature. Proteins were eluted with a linear NaCl gradient (0 to 400 mM in 70 min; flow rate, 1 ml/min) in buffer B. ACMS II isolated from *S. lividans* transformants was found to elute with 220 mM NaCl as did ACMS II isolated from *S. chrysomallus* (data not shown).

Thioester formation assay and unit definition. A 100-μl protein fraction was mixed with 3 μl of ^{14}C-labelled amino acid (100 μCi/ml)–2 μl of $MgCl_2$ (1 M)–15 μl of ATP (100 mM). After incubation for 30 min at 30°C, the reaction was stopped with 2 ml of 7% trichloroacetic acid (TCA) and protein was precipitated for 30 min on ice. The precipitate was collected on ME25 filters (Schleicher & Schuell), washed with 20 ml of TCA (7%), and dried, and protein-bound label was identified by liquid scintillation counting in a Wallac 1409 counter. One unit of ACMS II is the amount of enzyme which covalently binds 1 nmol of threonine in 30 min at 30°C.

SDS-PAGE analysis of ACMS II with covalently bound reaction intermediates. Engineered ACMS II (after Ultrogel-AcA-34 gel filtration; 0.04 U per reaction) was incubated for thioester formation with [^{14}C]threonine or [^{14}C]valine. Protein was precipitated with TCA and collected by centrifugation (15,000 × *g*, 30 min, 4°C). The precipitate was washed twice with 2 ml TCA (7%) and resuspended in a 50-μl solution of 15% (wt/vol) glycerol, 100 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM PMSF, and 1% sodium dodecyl sulfate (SDS). Control samples lacking ATP were prepared in parallel. Two microliters of 40% sucrose–0.25% bromphenol blue was added, and proteins were separated by SDS–4% polyacrylamide gel electrophoresis (PAGE). The Coomassie blue-stained gel was vacuum dried on a filter sheet (Whatman 3MM). Proteins with covalently bound label were identified by autoradiography (film NIF100; Konica).

Isolation of reaction intermediates from ACMS II and chromatographic analysis. Recombinant ACMS II purified from *S. lividans* (0.14 U after FPLC purification) was incubated for thioester formation with ^{14}C-labelled valine in the presence of 3 mM unlabelled threonine. Protein was precipitated with TCA and collected by centrifugation (15,000 × *g*, 30 min, 4°C). The precipitate was washed twice with 2 ml TCA (7%) and twice with 2 ml EtOH, dried at 37°C, and resuspended in 60 μl of formic acid. A 30-μl portion of the resuspended protein was mixed with 0.4 ml of performic acid (cleavage of thioester bonds); the remaining 30-μl portion was mixed with 0.4 ml of formic acid (control). After incubation for 20 h at 26°C, the samples were dried in a vacuum centrifuge, resuspended in 60 μl of formic acid, and analyzed on silica 60 thin-layer chromatography (TLC) plates (Merck) with the solvent system *n*-butanol–acetic acid–H_2O (4:1:1 [vol/vol/vol]). Labelled compounds were detected by autoradiography (R_f for [^{14}C]Val = 0.40). In the control reactions (no cleavage of thioester bonds) the label remained protein bound at the start position (data not shown). In the cleavage reaction, two labelled compounds with R_f values of 0.45 and 0.50 (expected to be Thr–D-[^{14}C]Val and Thr–L-[^{14}C]Val, respectively) were scraped from silica plates, extracted with 1 ml of 50% EtOH, dried in a vacuum centrifuge, and resuspended in 50 μl of H_2O. About 400 cpm of each compound (not UV detectable at 205 nm) was mixed with the authentic nonlabelled standard (UV absorbance of 0.4 at 205 nm) in a total volume of 100 μl and analyzed by reversed-phase high-pressure liquid chromatography (HPLC) on a SuperPac Pep-S column (Pharmacia). HPLC was performed at a flow rate of 0.5 ml/min with solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile) with the following profile of linear-gradient steps: 5 min, 0% solvent B; 40 min, 20% solvent B; 45 min, 100% solvent B. Fractions of 0.5 ml were collected, and labelled compounds were identified by liquid scintillation counting.

General methods for DNA manipulations. Standard procedures for DNA analysis were performed as described by Sambrook et al. (22). DNA fragments were purified from agarose gels with the JetSorb Extraction Kit 150 from Genomed. Plasmid DNA was isolated from *E. coli* as described by Birnboim and Doly (1); plasmids were isolated from *Streptomyces* as described by Hopwood et al. (10).

Cosmids and *E. coli* plasmids. Cosmids cosA1 and cosP1 are pHC79 derivatives (9) harboring size-fractionated fragments (both 32 kb) of genomic *S. chrysomallus* DNA obtained after partial *Sau*3A digestion (19). For restriction analysis, hybridization mappings of peptide synthetase activation domains, and sequencing, various fragments from cosA1 and cosP1 were subcloned in pTZ18 (Pharmacia), pSP72 (Promega), or pSL1180 (Pharmacia). Examples of these strategies are given in the figures and figure legends.

Construction of pACM5 for heterologous expression of *acmB*. pACM5 is a derivative of *Streptomyces* plasmid pIJ702 (11) in which the *melC1* gene of the melanine operon (*mel*) contained by the plasmid is replaced by *acmB*. The ATG start codon of *melC1* is contained in the unique *Sph*I restriction site of the plasmid and was used as an in-frame replacement. To generate a matching *Sph*I site at the start codon of *acmB*, the translation initiation start (underlined) of *acmB* (GGTTGAAACGTGTTC) was changed into GGCATGCATATGTTC by PCR. This resulted in two additional amino acids being attached to the amino terminus of the protein (change of MF- to MHMF-). For this modification, we synthesized a 0.5-kb gene fragment by PCR with primer A (5′-ATCGGAGGC ATGCATATGTTCGTCCGTCCTGATG-3′) and reverse primer B (5′-TCGG AGTCGCGGTACTTCTGATCGG-3′). Primer A binds to the translational start region of *acmB*, whereas primer B binds to a sequence located 548 bp downstream of the original GTG start codon. As can be seen from Fig. 1, the fragment encompasses a *Bgl*II site at position +50 and a *Sal*I site at position +265 of *acmB* (not shown). *Sph*I and *Sal*I digestion of the fragment thus resulted in a 265-bp *Sph*I-*Sal*I fragment which was cloned into pTZ18 cleaved with *Sph*I and *Sal*I. This generated plasmid pCR1. Control sequencing verified the correctness of the construct and sequence. Next, a 3.7-kb *Bgl*II-*Bam*HI fragment, ranging from the central *Bgl*II site in *acmB* to its next 3′-located *Bam*HI site (outside of *acmB*) and encompassing the stop codon, was isolated from cosA1 and inserted into *Bgl*II-*Bam*HI-cleaved pCR1. The resultant plasmid, pACMΔBg, harbors the engineered 5′ start of *acmB* fused to the large distal *Bgl*II-*Bam*HI fragment in the same orientation as in *acmB*. This cloned construct (Δ*acmB*) thus differs from the wild-type *acmB* in that the central 5.1-kb *Bgl*II fragment is deleted and the 5′ end is modified. pACMΔBg was cleaved with *Sph*I and *Bam*HI, and the isolated insert (Δ*acmB*) was ligated into pIJ702 cleaved with *Sph*I and *Bgl*II. After transformation into *S. lividans*, plasmid pACM3 was obtained. In pACM3, fusion of the *Bam*HI site to the *Bgl*II site of the plasmid destroys the *Bgl*II site. The missing central 5.1-kb *Bgl*II fragment of *acmB* was isolated from cosA1 and inserted into the unique *Bgl*II site of pACM3 (located in Δ*acmB*), which resulted in pACM5 containing the complete engineered *acmB*.

Oligonucleotide probes. Oligonucleotides were designed considering the codon usage of *Streptomyces* (31). From the N-terminal sequence of ACMS I (ADKWWGEQLLGRGDDGDLWAVSAAPVTRGELRA) (16), oligonucleotide acm1 (5′-TGGGGSGARCAGCTSCTSGGSCGSGGSGACGACGGSGAC CTSTGG-3′) was designed. For sequence determination of ACMS II, the protein was purified to homogeneity as described previously (26). The protein was digested with trypsin as described by Stone and Williams (28), and tryptic fragments were isolated by HPLC. The sequence of the tryptic fragment, TVFPE-VDGTPYQ(Q)R, was determined by automatic Edman degradation on an ABI gas phase sequencer. From this peptide sequence, oligonucleotide acm2 (5′-AC CGTCTTCCCGGAGGTCGACGGCACCCCGTACCAGCAGCG-3′) was designed. The following digoxigenin (DIG)-labelled oligonucleotides, derived from the consensus sequences of peptide synthetase core motifs 2, 5, and 6 of the activation domain, were kindly provided by S. Pelzer, University of Tübingen, Lehrstuhl für Mikrobiologie und Biotechnologie: core2, 5′-AGGCCTACATCA TCTACACCTCCGGCACGACGGGCAAGCCCAAGGG-3′; core5, 5′-CAGG TCAAGATCCGCGGCTACCGCATCGAGCTCGGCGAGATCGAG-3′; and core6, 5′-CTCGGCGGGCACTCCCTCAAGGCCT-3′.

DNA hybridization analysis. Oligonucleotides were diluted to about 100 ng/ml when used as probes for Southern analysis. Nucleic acids were transferred to Hybond-N membranes (Amersham) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and immobilized by UV cross-linking. For cosmid screening, *E. coli* colonies were transferred to membranes and DNA was released by alkaline treatment. Cosmid filters were hybridized with 3′ ^{32}P-labelled oligonucleotides (acm1 and acm2) at 65°C (in a solution containing 5× SSC, 0.02% SDS, 0.1% *N*-lauroylsarcosine, 1% blocking solution; Boehringer kit 1175041) and washed with 2× SSC–0.1% SDS at 65°C. Cosmids hybridizing with labelled probes were detected by autoradiography (film NIF100; Konica). Southern analysis with domain core probes was performed at 40°C, both for hybridization (20 h) and wash steps (twice for 30 min), with DIG nucleic acid detection kit 1175041 from Boehringer Mannheim. DIG-labelled nucleotides were detected with the anti-DIG-alkaline phosphatase conjugate supplied with the kit at 25°C for 20 to 30 min.

DNA sequencing analysis and computer analysis. The sequence of the *acmB* gene was determined with various fragments isolated from cosmid cosA1 and subcloned in pTZ18. The region between *acmA* and the single *Sph*I site in *acmB* (see also Fig. 1) was determined by *Taq* cycle sequencing (U.S. Biochemicals-Amersham sequencing kit US71001 or US78500) with universal oligonucleotide primers. The remaining part of *acmB* was sequenced by Eurogentec (Seraing, Belgium) by primer walking with dye-labelled dideoxy terminators (Applied

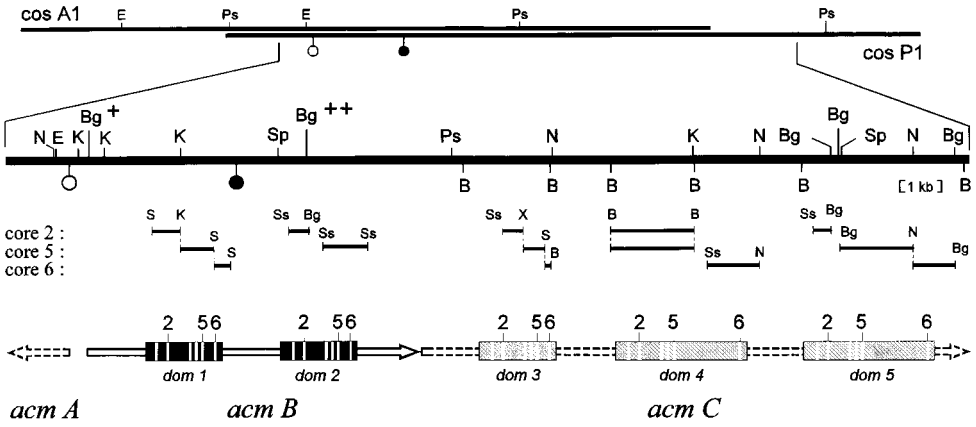

FIG. 1. Organization of the *acm* gene cluster. The region of *S. chrysomallus* DNA, cloned in cosA1 and cosP1, is shown at the top. The section that encompasses the actinomycin synthetase genes is shown enlarged, with all restriction sites of *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Kpn*I (K), *Not*I (N), *Sph*I (Sp), and *Pst*I (P) noted. Two *Bgl*II sites used for construction of pACM5 are marked (+ and ++). The location of the DNA sequence (5′-ATGGCCGATAAATGGTGGGGGGAACAAC TGCTGGGGCGCGGGGACGACGGTGATCTCTGGGCGGTCTCGGCCGCCCCGGTCACCCGGGGCGAGCTCCGCGCC-3′) coding for the N terminus of ACMS I is indicated by an open circle; the position of the sequence (5′-ACGGTGTTCCCCGAGGTCGACGGGACGCCGTACCAGCGG-3′) encoding the tryptic ACMS II fragment (subsequently identified at aa position 1124) is indicated by a solid circle. The mapped regions hybridizing with oligonucleotide probes designed from activation domain cores 2, 5, and 6 are shown below the enlarged section (S, *Sal*I; Ss, *Sst*I; X, *Xho*I). The ORF of *acmB* is indicated by a solid arrow, and the segments encoding the two activation domains of ACMS II are drawn as black boxes (*dom* 1 and 2); white stripes indicate the positions of domain core motifs 1 to 6. Gene *acmA* and putative gene *acmC* (dashed arrows) were sequenced partially. Sequencing includes the 5′ region of *acmA* up to the next *Eco*RI site and the three indicated fragments of *acmC* hybridizing with core probe 6. The domain-encoding segments assigned to *acmC* (*dom* 3 to 5) are drawn as shaded boxes. They are placed in that manner so that their core motifs fit the core hybridization mapping and are adjusted exactly to the identified localization of motif 6 (shown in Table 1). The indicated core motifs within *dom* 1 to 3 show the motif arrangement of a highly conserved standard activation domain (600 aa). In contrast, *dom* 4 and 5 are drawn as enlarged between motifs 5 and 6 to indicate activation domains with additional *N*-methyltransferase activity, as expected for ACMS III.

Biosystems; model 377). Sequence comparisons, multiple sequence alignments, and identity scores were computed with CLUSTAL V (8).

Radioisotopes and chemicals. L-[U-^{14}C]threonine (208 Ci/mol, 100 μCi/ml) and L-[U-^{14}C]valine (283 Ci/mol, 100 μCi/ml) were from DuPont. Authentic dipeptides used as standards for HPLC analysis were either from Bachem (L-Thr–L-Val) or were synthesized (L-Thr–D-Val) as described previously (27). The identity of L-Thr–D-Val was verified by mass spectrometry and amino acid analysis after acid hydrolysis.

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been assigned GenBank accession no. AF047717.

RESULTS

Molecular cloning of the actinomycin (*acm*) gene cluster. Genes for antibiotic biosynthesis in streptomycetes are usually clustered. A previous genetic analysis of mutants for actinomycin biosynthesis indicated a similar situation for the *acm* genes in *S. chrysomallus* (6). Nonpleiotropic *acm* mutations fell into one linkage group and were mapped to one interval of the *S. chrysomallus* chromosome. However, in all of the mutants the three ACMSs were present and most mutants were impaired in the production of antibiotic precursor 4-MHA (6). Since none of the mutants was suitable for cloning the peptide synthetase genes by complementation, we isolated these genes by screening a cosmid library of *S. chrysomallus* with oligonucleotides derived from protein sequences of ACMSs. Purified ACMS II was digested with trypsin, and from the peptide sequence of one tryptic ACMS II fragment [TVFPEVDGT PYQ(Q)R] oligonucleotide probe acm2 was designed. Hybridization screening with acm2 led to the isolation of two overlapping cosmids (cosA1 and cosP1) comprising a total stretch of 42 kb of genomic *S. chrysomallus* DNA as shown in Fig. 1. A second oligonucleotide probe, acm1, derived from the amino-terminal sequence of ACMS I (ADKWWGEQLLGRGD DGDLWAVSAAPVTRGELRA) (16), hybridized also with cosmids cosA1 and cosP1. This indicated that both actinomycin synthetase genes are located in the overlapping region of these cosmids. Detailed restriction analysis and hybridization mapping revealed that probe acm1 hybridized to a 0.6-kb *Eco*RI-*Kpn*I fragment and that probe acm2 hybridized to a 2.2-kb *Kpn*I-*Sph*I fragment. These fragments were further analyzed, and two DNA sequences were identified (see the legend for Fig. 1); the deduced amino acid sequences matched exactly with the two corresponding ACMS peptide sequences (with only one glutamine in the tryptic ACMS II sequence shown above).

Organization of the *acm* gene cluster. The DNA sequence coding for the N terminus of ACMS I was localized on a 0.6-kb *Eco*RI-*Kpn*I fragment, which allowed the mapping of the start and the orientation of the corresponding gene (*acmA*), as shown in Fig. 1. In order to locate the two expected activation domain-encoding regions of the ACMS II gene (*acmB*) and to see if there are any additional such regions on cosmids cosA1 and cosP1, combined restriction/hybridization mappings were performed with three different oligonucleotide probes, based on consensus signature sequences of peptide synthetase activation domains (cores 2, 5 [amino acid adenylation], and 6 [thioester formation]) (24, 25, 30). Sequence analyses of a number of acyl-adenylating enzymes have shown that their core motif sequences are less conserved than those in peptide synthetase domains. Therefore *acmA* was not expected to hybridize with any of these core probes under stringent conditions. In fact, these oligonucleotides hybridized to the region upstream of *acmA* but not to *acmA* itself or to its downstream-located region. An example of the core hybridization analysis, performed with a plasmid carrying the 5.1-kb *Bgl*II fragment upstream of *acmA*, is shown in Fig. 2. Three probes hybridized to three adjacent regions on the left side of the fragment in the motif order 2, 5, and 6. This order fits with the arrangement of the corresponding cores in a peptide synthetase activation domain (24). A fourth signal, seen with the core 2 probe, indicates the proximal portion of the next domain-encoding segment at the right site of the fragment. Extending this kind of mapping analysis to the whole cloned region upstream of *acmA* led to the detection of a total of five putative activation do-

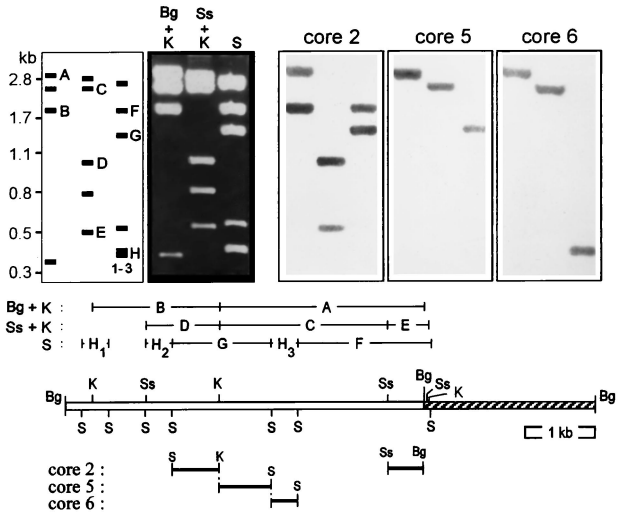

FIG. 2. Hybridization mapping of domain cores. Restriction fragments of plasmid pA1sub11, which is a pSP72 derivative carrying the 5.1-kb *Bgl*II fragment upstream of *acmA* (indicated by + and ++ in Fig. 1) in the *Bgl*II site of the pSP72 polylinker, were separated on a 1% agarose gel (second panel from left; Bg, *Bgl*II; K, *Kpn*I; S, *Sal*I; Ss, *Sst*I). Fragments were analyzed by Southern hybridization with DIG-labelled oligonucleotides designed from domain cores (motifs 2, 5, and 6; right three panels) as described in Materials and Methods. The Southern filters were prepared from three identical agarose gels (only one is shown) and hybridized exclusively with the indicated core probe to exclude the remains of previous stainings. The fragment pattern is schematically shown in the left panel, and all hybridizing fragments are indicated by letters A to H (three *Sal*I fragments of the same size [only one is hybridizing] are labelled 1-3). These fragments are aligned above the restriction map of pA1sub11 (the pSP72 portion is striped), which allows the mapping of the hybridizing regions for every core probe, as shown below the plasmid map.

main-encoding segments (*dom* 1 to 5; summarized in Fig. 1). These segments are separated from each other by more than 1 kb and are arranged in the same orientation, which is implied by the motif order of the hybridizing regions. The first two domain-encoding segments, *dom* 1 and *dom* 2, were analyzed in the course of sequencing *acmB* (see below); for *dom* 3 to 5 only the fragments hybridizing with core probe 6 (indicated in Fig. 1) were sequenced. All five segments were found to encode motif 6, which is the 4′-phosphopantetheine attachment site of peptide synthetases (see Table 1), and the missing hybridization signal of core probe 6 with segment *dom* 2 turned out to be only the result of less-conserved DNA sequence similarity. The presence of five domain-encoding segments arranged in the same orientation would be in agreement with the enzymatic activities of ACMS II and ACMS III, which activate two and three amino acids, respectively. The DNA sequence encoding the tryptic ACMS II peptide (TVFPEVDGTPYQR)

TABLE 1. Identified motifs for 4′-phosphopantetheine cofactor attachment

Segment in *acm* cluster[a]	Sequence of core 6	ACMS type[b]
*dom*1	D F F E L G G H S L	II
*dom*2	D F F A L G G D S I	II
*dom*3	D F F E L G G H S L	III
*dom*4	D F F E L G G H S L	III
*dom*5	D F F D L G G H S L	III

[a] Segments with respective motif positions are indicated in Fig. 1.
[b] Derived from sequencing *acmB* and the indicated fragments (Fig. 1) of the putative *acmC* gene region.

was mapped on the 2.2-kb *Kpn*I-*Sph*I fragment between *dom* 1 and *dom* 2, which implies that *acmB* spans these two domain-encoding segments. The region spanning segments *dom* 3 to 5 probably contains the gene coding for ACMS III (480 kDa) with an estimated size of about 13 kb (*acmC*).

Sequencing the ACMS II gene (*acmB*). Sequencing the region upstream of *acmA* revealed the presence of an open reading frame (ORF) of 7,833 bp starting 430 bp from the start codon of *acmA* and in the opposite orientation. The ORF shows the typical *Streptomyces* codon usage (31), with a 94% G+C content in the third codon position and an overall G+C content of 73%. The deduced protein has a size of 283.9 kDa, which fits well with the estimated size of ACMS II of 280 kDa (26). It contains two amino acid activation domains (schematically indicated in Fig. 1), as revealed by a sequence comparison with a number of peptide synthetase sequences (data not shown). The activation domain core motifs (motifs 1 to 6) (24), essential for ATP binding, amino acyladenylate formation, and covalent attachment of the 4′-phosphopantetheine cofactor, are located between amino acids (aa) 534 and 1015 in the first domain and between aa 1603 and 2064 in the second one. Four characteristic motifs for the epimerization function of peptide synthetases (motifs A to D) (24) are located in the C-terminal region distal to the second domain (between aa 2359 and 2493). This fits with the observed epimerization activity of ACMS II. A motif, which is thought to play a role in the peptide elongation reaction and/or acyl transfer (His motif or spacer motif) (3, 24), precedes both activation domains (at aa 140 and 1196), and a third His motif, thought to play a role in epimerization domains, is about 110 amino acids in front of the epimerase motifs (at aa 2250). This is in accordance with the established reaction mechanism of ACMS II in forming 4-MHA–threonine and 4-MHA–threonyl–D-valine (from the L-valine-containing diastereomer).

Functional expression of *acmB*. From a sequence analysis of *acmB*, the encoded protein was predicted to catalyze dipeptide formation and epimerization of the C-terminal amino acid. To demonstrate these activities, *acmB* was heterologously expressed in *S. lividans*. The 5′ end of *acmB* was engineered by PCR for expression from the *mel* promoter in *Streptomyces* plasmid pIJ702, as described in Materials and Methods. In the final construct, pACM5, *acmB* is inserted as an in-frame fusion to the ATG start codon of the *melC1* gene. In this construct, the recombinant ACMS II has two additional amino acids fused to the N terminus. *S. lividans* transformants harboring pACM5 did not produce melanine and were analyzed for the presence of engineered ACMS II. In crude extracts of these transformants, a protein in the range of 240 to 300 kDa, which was not seen in control strains harboring pIJ702, was detected (Fig. 3). After ammonium sulfate precipitation and gel filtration, protein fractions were tested for binding amino acid substrates as thioesters. Fractions containing the pACM5-encoded protein were able to form thioesters with threonine and valine (fractions 14 to 19 in Fig. 4), which are established substrates for ACMS II (13, 26). In a control experiment with proline, which is a substrate only for ACMS III, no thioester formation was detected (data not shown). Furthermore, in protein fractions of a control strain transformed with pIJ702 thioester formation was not detected either with threonine or with valine (data not shown). These results indicate that thioester formation detected in transformants harboring plasmid pACM5 is correlated with the presence of the engineered ACMS II.

To clearly assign thioester formation to the plasmid-encoded synthetase, protein fractions showing this activity were analyzed by SDS-PAGE after incubation with labelled substrates (Fig. 5A). Both radioactive amino acids, threonine and valine,

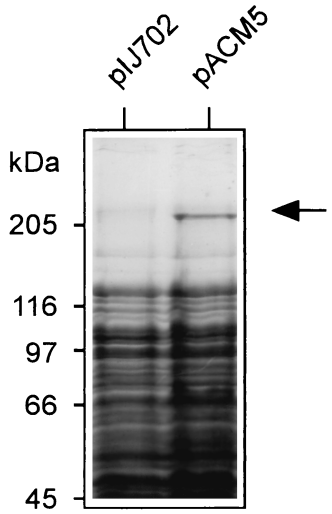

FIG. 3. SDS-PAGE analysis of engineered ACMS II expressed from pACM5 in *S. lividans*. Cells harboring plasmid pIJ702 (control) or pACM5 (expression of *acmB*) were grown as described in Materials and Methods and broken by sonification. Proteins of total crude extracts were separated on a 5% polyacrylamide gel and stained with Coomassie blue. The arrow indicates ACMS II.

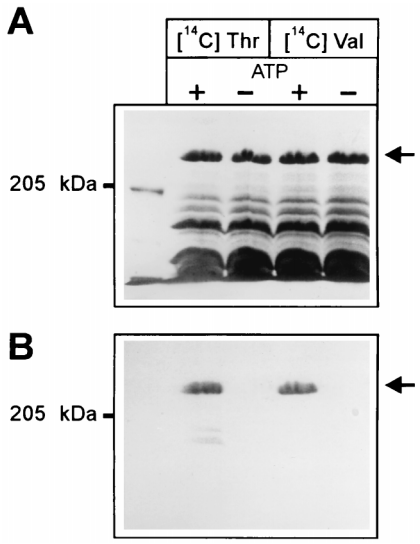

FIG. 5. Amino acid substrates covalently bound to engineered ACMS II as thioesters. (A) Coomassie blue-stained 4% polyacrylamide gel of Ultrogel-AcA-34-purified ACMS II after incubation with [^{14}C]threonine (left) or [^{14}C]valine (right) in the presence (+) or absence (−) of ATP as described in Materials and Methods. (B) Autoradiograph of the gel shown in panel A.

clearly labelled the engineered ACMS II in an ATP-dependent fashion (Fig. 5B). The 4′-phosphopantetheine content of the enzyme was not determined.

To address the additional functions of the synthetase, such as peptide bond formation and peptide epimerization, the FPLC-purified enzyme was incubated with [^{14}C]valine and unlabelled threonine in the presence of ATP. After completion of the reaction, enzyme-bound material was isolated and separated by TLC (Fig. 6A). In the presence of both substrate amino acids (Fig. 6A, lane 2) two new additional compounds, which were not seen with valine alone were detected (Fig. 6A, lane 1). In previous investigations with ACMS II isolated from *S. chrysomallus*, a similar formation of two compounds with R_f values slightly higher than those of valine was observed; the two compounds have been identified as Thr–L-Val and Thr–D-Val (27). To demonstrate that the products synthesized by plasmid-encoded ACMS II are identical to Thr–L-Val and Thr–D-Val, the two compounds were further analyzed by HPLC and compared with corresponding nonlabelled standards (Fig. 6B). HPLC analysis clearly shows that the engineered ACMS II is able to catalyze the formation of a threonyl–L-valine dipeptide, which is epimerized to threonyl–D-valine.

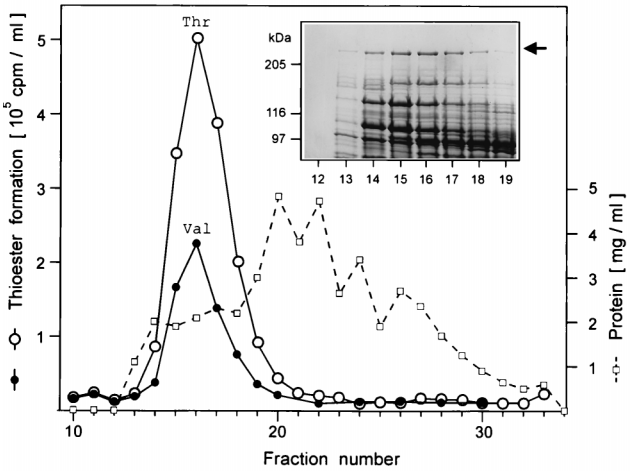

FIG. 4. Gel filtration of the engineered ACMS II on Ultrogel-AcA-34. Detection of thioester formation with [^{14}C]threonine (open circles) or [^{14}C]valine (solid circles) was performed as described in Materials and Methods. The inset shows the results of an SDS-PAGE analysis (5% polyacrylamide; Coomassie blue-stained) of fractions (15-μl aliquots) in which enzymatic activity was detected. The protein concentration is shown by the dashed curve. The arrow indictaes ACMS II.

DISCUSSION

Actinomycin half molecules (4-MHA pentapeptide lactones) are assembled by two peptide synthetases (ACMS II and III) in conjunction with a 4-MHA–adenylate ligase (ACMS I). By using oligonucleotides derived from partial peptide sequences of ACMS I and II we cloned a gene cluster from *S. chrysomallus* containing the corresponding genes *acmA* and *acmB*, respectively. Sequencing *acmB* revealed that it codes for a peptide synthetase of 284 kDa with two amino acid activation domains and one epimerization domain. A hybridization analysis with oligonucleotide probes coding for signature sequences of peptide synthetase activation domains indicated the presence of a further peptide synthetase gene with three domain-encoding segments downstream of *acmB* (Fig. 1). Since ACMS II incorporates the first two amino acids of the 4-MHA pentapeptide lactone and ACMS III incorporates the remaining three, this second peptide synthetase gene most probably is the gene encoding ACMS III (*acmC*).

Heterologous expression of *acmB* in *S. lividans* yielded a functionally active peptide synthetase specifically activating L-threonine and L-valine as thioesters. Moreover, it catalyzed the formation of the threonyl–L-valine and threonyl–D-valine dipeptides, as does ACMS II from *S. chrysomallus*. These catalytic activities are in agreement with the sequence data and leave little doubt that the *acmB* gene product is ACMS II. The ACMS II sequence showed the greatest similarity to pristinamycin I synthetase C (SnbC) from *Streptomyces pristinaespiralis* (2) (50% identity over the complete sequence of the two en-

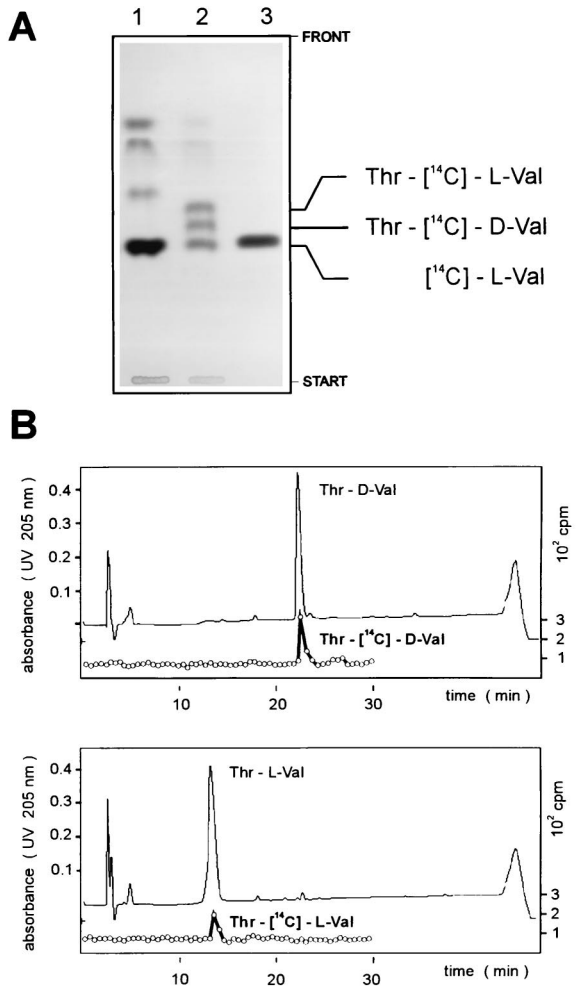

FIG. 6. Formation of threonyl–L-valine and threonyl–D-valine catalyzed by engineered ACMS II. (A) Reaction intermediates from thioester formation, covalently bound to ACMS II, were cleaved off with performic acid and analyzed on TLC silica plates as described in Materials and Methods. Thioester formation was performed with L-[^{14}C]valine (lane 1) or with L-[^{14}C]valine in the presence of unlabeled L-threonine (lane 2). The reference was L-[^{14}C]valine (lane 3). Labeled compounds were identified by autoradiography. (B) The two compounds, expected to be Thr–D-[^{14}C]Val and Thr–L-[^{14}C]Val were isolated from the silica plate shown in panel A and analyzed by HPLC as described in Materials and Methods. About 400 cpm of each compound (not UV detectable) was mixed with an unlabeled reference dipeptide (UV detection at 205 nm).

zymes). Pristinamycin I, an acyl hexapeptide lactone belonging to the group of the mikamycin B antibiotics, has striking structural similarity to the actinomycin half molecules (18, 23). Instead of 4-MHA, mikamycin B antibiotics contain 3-hydroxypicolinic acid as an acyl side group. However, from the number and similarity of amino acid residues in their hexa- or heptapeptide lactone rings, mikamycin B antibiotics can be regarded as elongated versions of the 4-MHA pentapeptide lactone structure (23). It was suggested that aromatic acyl peptide lactones such as the mikamycins and the actinomycin half molecules are synthesized by similar sets of enzymes (5, 14, 16, 23). In fact, Thibaut et al. (29) recently showed that pristinamycin I is also synthesized by three synthetases, SnbA, SnbC, and SnbD. By comparison, these have been postulated to function like their corresponding ACMS I, II, and III analogs except that SnbD activates four amino acids compared to the three activated by ACMS III. The sequence similarity between ACMS II and SnbC is thus consistent with the similar roles of the two enzymes, i.e., activation of the first two amino acids of the peptide lactone rings, acyl dipeptide formation, and epimerization.

The results of previous biochemical investigations of 4-MHA–threonine and 4-MHA–threonyl–LD-valine formation catalyzed by ACMS II in conjunction with ACMS I suggested that 4-MHA is covalently bound as a thioester to a phosphopantetheine cofactor presumed to reside on ACMS II. However, inspection of the sequence of ACMS II presented here revealed only two 4′-phosphopantetheine attachment sites in the protein sequence (one in the threonine activation domain and the other in the valine activation domain). This may point to an additional factor providing the missing third 4′-phosphopantetheine cofactor required for the formation of the 4-MHA–Thr peptide bond. This factor could have escaped detection in enzyme preparations due to low concentration or small size. Interestingly, Gehring et al. (4) characterized an as yet unsuspected acyl carrier domain as a component of the enterobactin biosynthesis system in *E. coli*. Enterobactin is a cyclic trilactone composed of 2,3-dihydroxybenzoyl–serine (2,3-DHB–serine) units. The 2,3-DHB–serine unit is assembled by enzymes EntE and EntF (20, 21), which have been suggested to function in a manner similar to that of ACMS I and II or SnbA and SnbC in the formation of 4-MHA–threonine or 3-hydroxypicolinic acid–threonine. The respective acyl carrier domain is part of isochorismate lyase (EntB) involved in 2,3-DHB synthesis and was shown to be pantetheinylated by a specific enzyme. Interestingly, EntE activates 2,3-DHB as adenylate and acylates EntB with 2,3-DHB. Gehring et al. (4) postulate that the thioester-bound 2,3-DHB residue is subsequently transferred from EntB to the amino group of serine covalently bound to the 4′-phosphopantheine arm of EntF, yielding 2,3-DHB–serine. A similar mechanism for the initiation of actinomycin half-molecule synthesis is imaginable if a comparable acyl carrier protein would interact with ACMS I and II. To clarify the exact mechanism, more detailed investigations, both on the enzymatic and genetic levels, have to be performed. The in vitro studies presented have shown that heterologous expression of the ACMS II gene yielded a synthetase which maintained its specific activities, not only with respect to amino acid activation but also with respect to dipeptide formation and epimerization. This important finding and the successful cloning of the *acm* gene cluster should provide the future basis to investigate ACMS interactions by a stepwise rebuilding of the system in a heterologous host and to get more insight into the mechanism of acyl peptide lactone synthesis.

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