Sequencing and Analysis of the Biosynthetic Gene Cluster of the Lipopeptide Antibiotic Friulimicin in *Actinoplanes friulienis*\( ^{\text{a}} \)

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*Actinoplanes friulienis* produces the lipopeptide antibiotic friulimicin, which is a cyclic peptide with one exocyclic amino acid linked to a branched-chain fatty acid acyl residue. The structural relationship to daptomycin and the excellent antibacterial performance of friulimicin make the antibiotic an attractive drug candidate. The complete friulimicin biosynthetic gene cluster of 24 open reading frames from *A. friulienis* was sequenced and analyzed. In addition to genes for regulation, self-resistance, and transport, the cluster contains genes encoding peptide synthetases, proteins involved in the synthesis and linkage of the fatty acid component of the antibiotic, and proteins involved in the synthesis of the nonproteinogenic amino acids pipercolinic acid, methylaspartic acid, and 2,3-diaminobutyric acid. By using heterologous gene expression in *Escherichia coli*, we provide biochemical evidence for the stereoselective synthesis of L-pipecolinic acid by the deduced protein of the lysine cyclodeaminase gene *pip*. Furthermore, we show the involvement of the *dabA* and *dabB* genes in the biosynthesis of 2,3-diaminobutyric acid by gene inactivation and subsequent feeding experiments.

In recent years, the sequences of numerous gene clusters involved in the synthesis of many secondary metabolites have become available for comparison and exploitation. The programmed manipulation of genes encoding enzymes in the biosynthetic pathways offers promise for redesigning existing antibiotic structures to create antibiotics with new activities and the ability to overcome bacterial resistance (50). Therefore, each newly analyzed gene cluster represents a new tool for combinatorial biosynthesis and can provide information about the synthesis of unusual building blocks, such as nonproteinogenic amino acids, acyl residues, and sugar moieties. An interesting group of secondary metabolites for such experiments seems to be bioactive lipopeptides isolated from streptomycetes. So far, only a few biosynthetic gene clusters corresponding to these lipopeptides have been isolated and characterized, such as the clusters for calcium-dependent antibiotic (CDA) from *Streptomyces coelicolor* (17), daptomycin from *Streptomyces roseosporus* (26), and A54145 from *Streptomyces fradiae* (24). By targeted modification and gene exchange, it is possible to generate new lipopeptide structures (12, 25).

Another member of this group of secondary metabolites is the antibiotic friulimicin that is produced by the actinomycete *Actinoplanes friulienis*. This compound is highly active against multidrug-resistant gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* strains (4). The biosynthesis of this lipopeptide is catalyzed by nonribosomal peptide synthetases (NRPS) (15).

The eight bioactive lipopeptides isolated from *A. friulienis* (4) consist of 10 amino acids that form a ring structure with one exocyclic amino acid linked to an acyl residue of various chain lengths. The acyl residue is a branched-chain fatty acid with a Δcis3 double bond. The structures of four of these lipopeptides are identical to those of known peptide antibiotics of the amphomycin group and possess aspartic acid at the exocyclic position. The other four lipopeptides have asparagine at this position and are known as friulimicins A to D. The peptide component of these friulimicins is characterized by unusual amino acids, such as L-threo- and D-erythro-2,3-diaminobutyric acid, L-pipecolinic acid, and L-threo-β-methylaspartic acid (48) (Fig. 1). The peptide also contains the conserved amino acid sequence DXDG, which is a putative calcium-binding site in the nonribosomally synthesized lipopeptide A54145 (24), daptomycin, and CDA and in the ribosomally assembled calmodulin (11) (Fig. 2).

Here we describe the sequencing and analysis of the complete gene cluster for the biosynthesis of friulimicin from *A. friulienis*. We also describe in detail the functions of enzymes involved in the synthesis of the unusual amino acids 2,3-diaminobutyric acid and pipercolinic acid.

**MATERIALS AND METHODS**

*Strains and plasmids.* *A. friulienis* HAG010964 (4) was studied. *Bacillus subtilis* ATCC 6633 (ATCC, Manassas, VA) was used as a friulimicin-sensitive indicator organism. *Escherichia coli* TOP 10 (Invitrogen, Karlsruhe, Germany) was used for cloning, and *E. coli* BL21(DE3)pLysS (Invitrogen) and *Streptomyces lividans* TK23 (18) were used for heterologous expression. The methylase-negative strain *E. coli* ET21267/pUB307 (9) was used as the donor strain for intergeneric conjugation.

Plasmid vectors used in this study were pMosBlue (Amersham Biosciences, Freiburg, Germany), pBluescript (Stratagene, Amsterdam, The Netherlands), pLutmus28 (New England BioLabs, Frankfurt, Germany), pOK12 (49), and...
pUC19 (52). The cosmid library for *A. friuliensis* was constructed with pOJ436 (6). The vector pRSETB (Invitrogen) and the streptomycete shuttle (6). The unusual amino acids described in this study are Asp, methylaspartic acid; Asp, aspartic acid; Gly, glycine; Val, valine; Asn, asparagine; Dab, diaminobutyric acid; Pip, pipecolinic acid; MeGlu, 3-methylglutamic acid; Orn, ornithine; Pip, pipecolinic acid; Sar, sarcosine.

The unusual amino acids described in this study are indicated with numbers at the ends of the amino acid abbreviations. Abbreviations for unusual amino acids are as follows: Asn(OH), hydroxyasparagine; 2,3Dab, 2,3-diaminobutyric acid; HPG, hydroxyphenylglycine; Kyn, kynurenine; MeAsp, methylaspartic acid; MeGlu, 3-methylglutamic acid; Orn, ornithine; Pip, pipecolinic acid; Sar, sarcosine.

### DNA sequencing and analysis
Cosmid 4E08 was shotgun sequenced. Overlapping DNA fragments of cosmids 18M80 and 5P02 were detected by Southern hybridization, subcloned, and sequenced. Sequences were analyzed with Clone Manager 7 (Scientific and Educational Software, Cary, NC), Artemis (33), Clustal X (47), GeneDoc (29), and the NRPS-PKS database (3). Open reading frames (ORFs) were analyzed using the *Actinoplanes teichomyceticus* codon usage table (28). Functional assignments were made using the BLAST program (2) to compare the deduced gene products with proteins of known functions listed in the NCBI database. Cosmids 18M80, 4E08, and 5P02 cover the regions of the genes (bp 115 to 156 of *orfO* to *psbB*, *lipA* to *lipE*, and *putC* to *orf33*, respectively (Fig. 3).

### Construction of plasmids pCMF11 and pCMF10
For the construction of the gene disruption mutants, internal fragments of approximately 600 bp of the *orfO* and *orfAP* genes were obtained by PCR using the primer pair O1/O2 for *orfO* and the primer pair AP1/AP2 for *orfAP* (Table 1). The fragments were cloned into HindIII-EcoRI-digested vector pDS401, resulting in pCMF11 and pCMF10, respectively. Gene disruption mutants were constructed by intergeneric conjugation.

### Construction of plasmids pCMOPApra and pCMTDApra
For gene replacement mutagenesis of *orfP* and *orfTD*, plasmids pCMOPApr and pCMTDApr were used. The corresponding genes were inactivated by replacing internal fragments of the genes (bp 115 to 156 of *orfP* and bp 139 to 524 of *orfTD*) with the apramycin/ermE*p* resistance cassette *aprP* (16). To lessen possible polar effects, *aprP* was inserted in the transcriptional direction of the affected genes. The inactivated genes in combination with DNA fragments of 1 to 2 kb from the flanking regions were subsequently cloned into the vector pK18mob (37), resulting in plasmids pCMOPApra and pCMTDApra, respectively. Gene replacement mutants were generated by intergeneric conjugation.

### Construction of the *A. friuliensis pip* mutant
The pip gene was inactivated by gene replacement as follows: a 7,363-bp MluI fragment from cosmid 5P02, carrying the genes *psbD* to *orfP* (Fig. 3), was cloned into pOK12. A 1,950-bp NcoI fragment of the flanking region upstream of *pip* and a 1,625-bp XhoI fragment of the flanking region downstream of *pip* from the resulting plasmid were subcloned into pOK12 and pLitmus28, respectively. The two fragments were cut out using EcoRI XhoI and HindIII Xbal, respectively, and ligated with HindIII-EcoRI-digested vector pK18mob in one step. The apramycin resistance cassette *aprP* was isolated from pKC505 by EcoRI-PstI digestion, ligated to pLitmus28, and cut out using XbaI and SpeI. The cassette was then integrated into the XbaI restriction site of the pK18mob resistance cassette (16), and pSET152 (6) were used for intergeneric conjugation.

### Construction of the *A. friuliensis pip* mutant
A 4,201-bp Stul-ScaI fragment of cosmid 18M80 was inserted into pK18mob in one step. The apramycin resistance cassette was inserted from pK18mob to putC (37), resulting in pCMF11 and pCMF10. For the construction of the *dabA* and *dabB* mutants, plasmids pCMO3Apra, used for the construction of the *dabB* knockout mutant, was generated as follows: a 4,201-bp Stul-ScaI fragment of cosmid 18M80, carrying the genes *regA* to *dabC*, was used for intergeneric conjugation between *E. coli* ET1520/pUB307 and *A. friuliensis*. Apramycin-resistant and kanamycin-sensitive double-crossover mutants were selected.

### Construction of the *dabB* and *dabB* mutants
Plasmid pCMO3Apra, used for the construction of the *dabB* knockout mutant, was generated as follows: a 4,201-bp Stul-ScaI fragment of cosmid 18M80, carrying the genes *regA* to *dabC*, was used for intergeneric conjugation between *E. coli* ET1520/pUB307 and *A. friuliensis*. Apramycin-resistant and kanamycin-sensitive double-crossover mutants were selected.

### Construction of plasmids pCMOPApra and pCMTDApra
For gene replacement mutagenesis of *orfP* and *orfTD*, plasmids pCMOPApr and pCMTDApr were used. The corresponding genes were inactivated by replacing internal fragments of the genes (bp 115 to 156 of *orfP* and bp 139 to 524 of *orfTD*) with the apramycin/ermE*p* resistance cassette *aprP* (16). To lessen possible polar effects, *aprP* was inserted in the transcriptional direction of the affected genes. The inactivated genes in combination with DNA fragments of 1 to 2 kb from the flanking regions were subsequently cloned into the vector pK18mob (37), resulting in plasmids pCMOPApra and pCMTDApra, respectively. Gene replacement mutants were generated by intergeneric conjugation.

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For gene replacement mutagenesis of *orfP* and *orfTD*, plasmids pCMOPApr and pCMTDApr were used. The corresponding genes were inactivated by replacing internal fragments of the genes (bp 115 to 156 of *orfP* and bp 139 to 524 of *orfTD*) with the apramycin/ermE*p* resistance cassette *aprP* (16). To lessen possible polar effects, *aprP* was inserted in the transcriptional direction of the affected genes. The inactivated genes in combination with DNA fragments of 1 to 2 kb from the flanking regions were subsequently cloned into the vector pK18mob (37), resulting in plasmids pCMOPApra and pCMTDApra, respectively. Gene replacement mutants were generated by intergeneric conjugation.

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Radioactive digoxigenin DNA labeling and detection kit from Roche (Mannheim, Germany) following the procedure described by Heinzelmann et al. (15).

The apramycin/ermE*p resistance cassette aprP was isolated from vector pEH13 (16) after Stul-EcoRV digestion and integrated into the DraIII-restricted pK18mob derivative in the transcriptional direction of dabA, resulting in plasmid pCMO3ApraN.

The dabB knockout mutant was generated as follows: a 6,651-bp Stul-Eco47III fragment was isolated from cosmid 18M80 and cloned into pMosBlue. From the resulting plasmid, a 1,627-bp BamHI-NcoI fragment covering the upstream flanking region and a 1,711-bp SacI fragment covering the downstream flanking region of dabB were subcloned into pLtmsu28. These fragments were cut out from the corresponding plasmids using PstI-XbaI and AacC6I-XbaI, respectively, and cloned together with pK18mob-digested pK18mob in one step. The aprP cassette was isolated from vector pEH13 (16) after SpeI digestion and integrated into the XbaI-restricted pK18mob derivative in the transcriptional direction of dabB, resulting in plasmid pCMO3Apra. The plasmids pCMO3Apra and pCMO4Apra were used for the construction of the dabA and dabB knockout mutants with intergeneric conjugation.

**Intergeneric conjugation between E. coli and A. friuliensis.** All gene inactivation mutants of A. friuliensis were generated by intergeneric conjugation by following the procedure described by Heinzelmann et al. (15).

**Southern hybridization.** Southern hybridization was performed using the non-radioactive digoxigenin DNA labeling and detection kit from Roche (Mannheim, Germany).

**TABLE 1. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Size&lt;sup&gt;b&lt;/sup&gt; (bp)</th>
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<tr>
<td>O1</td>
<td>5'-AAAAAGCTTCCGAAGATCCGCTGAGGGATCAGGC-3'</td>
<td>626</td>
</tr>
<tr>
<td>O2</td>
<td>5'-AACCTGAGTCCGCGCCGCTGAGGGATCAGGC-3'</td>
<td>626</td>
</tr>
<tr>
<td>AP1</td>
<td>5'-AAAAAGCTTTGCACAGAGATCCGAAGATCCGCTGAGGG-3'</td>
<td>629</td>
</tr>
<tr>
<td>AP2</td>
<td>5'-AACCTGAGTCCGCGCCGCTGAGGGATCAGGC-3'</td>
<td>1,025</td>
</tr>
<tr>
<td>PI1</td>
<td>5'-AGATGTTAGTATGATATGGATCTGCTGAGGC-3'</td>
<td>3'</td>
</tr>
<tr>
<td>PI2</td>
<td>5'-AGATGTTAGTATGATATGGATCTGCTGAGGC-3'</td>
<td>3'</td>
</tr>
</tbody>
</table>

<sup>a</sup> Restriction sites used for cloning are underlined.

<sup>b</sup> Expected size of the amplification product.

Friulimicin production bioassay. The production of friulimicin by A. friuliensis and the mutants was tested in a bioassay using friulimicin-sensitive Bacillus subtilis ATCC 6633. Strains were cultivated in Bacto tryptic soy broth (Becton, Dickinson and Company, Sparks, MD) for 7 days, and cultures were homogenized and spread onto the surface of M65 defined medium agar plates (the recipe is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). After 9 days of incubation at 28°C, plugs of equal sizes were cut out and applied to Bacillus subtilis test medium (1). The plates were incubated overnight at 37°C, and antibiotic production was evaluated by the detection of zones of growth inhibition around the agar blocks.

**Cosynthesis of friulimicin by pairs of mutants.** Pairs of different mutants were spread in smears 2 mm apart and approximately 1 cm in width on M65 agar plates. After 9 days of incubation at 28°C, strips of 2 cm perpendicular to the smears were cut out and used for antibiotic detection as described above. The shape of the growth inhibition zone was observed, and the two mutants were classified as the converter and the secretor.

**Cloning of pip for heterologous expression and genetic complementation.** The pip gene was isolated by PCR using the primer pair PI1/PI2 (Table 1), and the PCR product was cloned into pMosBlue. The resulting plasmid was digested with BglII and EcoRI, and the pip fragment was cloned into BglII-EcoRI-digested pRSETB, resulting in pRF37. This plasmid carries the sequence for the production of an N-terminal-His fusion protein and was used for heterologous expression.

For integration into the genome of the A. friuliensis pip knockout mutant, pRF37 was digested with XbaI and EcoRI, yielding a fragment encoding the His-tagged protein and the ribosome-binding site of pRSETB. The fragment was cloned downstream of the emrE*p sequence of pEM4. The expression construct together with the emrE*p sequence was cut out with HindIII and EcoRI and cloned into pOK12. In order to obtain a different selection marker for the transfer into the apramycin-resistant A. friuliensis mutants, the hygromycin resistance cassette hyg from vector pHP1H9024 was cut out with Dral and inserted into EcoRV-digested pSET152. The plasmid obtained, pSET1H9024, was linearized with BamHI, and the overhanging ends were filled in using the Klenow fragment. The expression construct from the pOK12 derivative was isolated as an EcoRV-Stul fragment and ligated into the linearized pSET1H9024, containing the 43631 attachment site for genomic integration.

**Construction of vector pSET345XEHyg for the genetic complementation of the dabA and dabB knockout mutants.** The above-described 6,651-bp Stul-Eco47III fragment from cosmid 18M80 in pMosBlue carries the genes regA*, expB, dabA, dabB, dabC, and pstA*. This fragment was isolated by XbaI-EcoRI.
digested and inserted into pEM4 downstream of the ermE’p sequence. The entire construct was further processed for cloning into pSETHyg as specified for the genetic complementation of pip (see above), yielding pSET345XEHyg, which carries the hygromycin resistance cassette and the F’C31 attachment site.

Heterologous expression of pip in E. coli BL21(DE3)pLys and purification of the His-tagged protein. E. coli BL21(DE3)pLys was transformed with plasmid pRF37 (see above) or plasmid pRESETB. Luria-Bertani medium (34) (150 ml) was inoculated with 1.5 ml of an overnight culture of a particular strain and incubated at 37°C with shaking at 180 rpm. Expression was induced with 0.8 mM IPTG (isopropyl-p-D-thiogalactopyranoside) when the culture reached an optical density at 600 nm of 0.5 to 0.6, and the culture was further incubated for 2 h. The protein was purified under both denaturing and native conditions from cells in 50 ml of the culture by following the procedure described by QIAAGEN (Hilden, Germany).

Preparation of the ethanol-soluble fractions from E. coli BL21(DE3)pLys(pRF37) and E. coli BL21(DE3)pLys(pRESETB). The pellets of freshly harvested and washed cells from 150 ml cultures of E. coli BL21(DE3)pLys(pRF37) and E. coli BL21(DE3)pLys(pRESETB) were extracted with 10 ml of 80% ethanol per gram of cell material with shaking at 180 rpm at 37°C for 1 h. The extracts were collected. The insoluble material was extracted with the same amount of 80% ethanol under the same conditions for 1 h and then incubated in a water bath at 95°C for 10 min. The extracts were pooled and evaporated to dryness; the residues were dissolved in 1.5 ml of distilled water.

HPLC analysis of piperocine acid produced in the heterologous host E. coli. Pipericine acid in the ethanol-soluble fractions (12 ml each) from E. coli BL21(DE3)pLys(pRF37) and E. coli BL21(DE3)pLys(pRESETB) was detected by reversed-phase high-performance liquid chromatography (HPLC) using an MSD 1100 HPLC system (Agilent Technologies, Boeblingen, Germany) and a 125-by-4-mm steel column containing Purospher RP-18. Amino acids were detected by staining with 0.2% (wt/vol) ninhydrin as a solvent. Amino acids were detected by staining with 0.2% (wt/vol) ninhydrin in 80% ethanol. 2,3-Diaminobutyric acid was also detectable by reversed-phase high-performance liquid chromatography (HPLC) using an MSD 1100 HPLC system (Agilent Technologies, Boeblingen, Germany) and a 125-by-4-mm steel column containing Purospher RP-18 (5 μm; Merck, Darmstadt, Germany). Compounds were eluted with a gradient of solvent A (0.1% TFA in H2O) and solvent B (methanol) with an elution gradient of 45% solvent B in solvent A to 95% solvent B in solvent A in 20 min.

Preparation of the ethanol-soluble fraction from friulimicin. E. coli BL21(DE3)pLYS(pPSETB) were extracted with 10 ml of 80% ethanol per gram of cell material with shaking at 180 rpm at 37°C for 20 min. The extracts were collected. The insoluble material was extracted with the same amount of 80% ethanol under the same conditions for 1 h and then incubated in a water bath at 95°C for 10 min. The extracts were pooled and evaporated to dryness; the residues were dissolved in 1.5 ml of distilled water.

HPLC analysis of pipecolinic acid produced in the heterologous host E. coli. The ethanolic-soluble fraction (20 μl) was applied in two lanes on a preparative silica gel 60 plate (ALUGRAMSIL G/UV 254; Macherey-Nagel, Duren, Germany). Compounds were separated with acetone-methanol-water (10:2:2, vol/vol/vol). One lane was stained with 0.2% (wt/vol) ninhydrin in 80% ethanol, and the material in the corresponding area containing pipecolic acid in the undesired lane was scraped off the plate and suspended in 50 ml of 80% ethanol. Silica gel was removed by filtration through a Membrex 13PES filter (pore size, 0.45 μm; MembraPure, Lorzweiler, Germany). The solvent was evaporated to dryness, and the remaining residue was dissolved in 50 μl of distilled water. Ten micro-liters of this solution, 5 μl of ρ-aminocapric acid, and 5 μl of ρ-aminocaprylic acid (Sigma, Munich, Germany) were applied to a chiral plate (CHIRALPLATE for enantiomeric resolution by thin-layer chromatography (TLC) 10 by 20 cm; Macherey-Nagel) to determine the amino acid configuration. Compounds were separated using the same solvent system and staining as described above. The densities of the spots were determined by scanning the TLC plates with Gene Tools analysis software (SynGene, Cambridge, United Kingdom). The l and d forms of pipecolic acid showed a characteristic violet color after ninhydrin staining and were easily distinguished by their Rf values of 0.62 and 0.58, respectively.

Isolation of 2,3-diaminobutyric acid from friulimicin. Friulimicin D (0.8 g) was hydrolyzed at 110°C in 45 ml of 6 N HCl for 17 h under an argon atmosphere. The fatty acids were removed by extraction with 50 ml of ether, and the HCl was removed by evaporation in vacuo. The remaining residue was dissolved in 5 ml of distilled water and applied to a column of Dowex 50WX4 cation exchange resin (1 by 20 cm). Compounds were eluted with 30 ml of 12 N HCl (fraction 1.2N) followed by 30 ml of 2 N HCl (fraction 2N) (7). Fraction 2N was evaporated to dryness, washed with 10 ml of methanol, and evaporated again. The fraction was dissolved in 2 ml of distilled water. 2,3-Diaminobutyric acid was detected by TLC using cellulose MN 300 plates (POLYGRAMCEL 300; Macherey-Nagel) and methanol–H2O–6 N HCl–pyridine (20:6.5:1:2.5, vol/vol/vol/vol) as a solvent. Amino acids were detected by staining with 0.2% (wt/vol) ninhydrin in 80% ethanol. 2,3-Diaminobutyric acid was also detectable by reversed-phase HPLC-mass spectroscopy using a 125-by-4-mm steel column containing Lichrospher 100 RP-18 gel (5 μm; Merck) and mobile phases of solvent A (0.1% formic acid in H2O) and solvent B (methanol) with an elution gradient of 45% solvent B in solvent A to 95% solvent B in solvent A in 20 min.

Overexpression of exp1 in Streptomyces lividans TK23. A 1,007-bp SalI fragment of cosmID 18M80 carrying the expA gene was cloned into Sall-digested pLItmus28. The resulting plasmid was digested with SpeI and EcoRI, and the resulting fragment was inserted into XbaI-EcoRI-digested pEM4 downstream of the ermE’p sequence. The resulting plasmid pEM4ExpA was used for the polyclonal glycol-mediated protoplast transformation of Streptomyces lividans TK23 by following the procedure described by Hopwood et al. (18).

Determination of the MIC. Transformsants of Streptomyces lividans TK23 were cultivated for 7 days at 30°C on Luria-Bertani agar plates containing friulimicin at the following concentrations: 0, 0.25, 0.5, 1.0, 5.0, 10, 25, 50, 75, 100, and 150 μg ml⁻¹. The MIC was determined by the span from the concentration with weak detectable growth to the concentration with complete growth repression.

Nucleotide sequence accession number. The nucleotide sequence data reported here were deposited in the EMBL data library under accession no. AJ488769.

RESULTS AND DISCUSSION

Organization of the friulimicin biosynthetic gene cluster. Heinzelmann and coworkers previously identified a 15.4-kb DNA fragment of the A. friuliiensis friulimicin biosynthetic gene cluster located on cosmID 18M80 (14, 15). The missing upstream section of the gene cluster was identified by further sequence analysis of this cosmID. In order to complete the sequencing of the friulimicin gene cluster downstream of the ORFs contained on 18M80, a cosmid library for A. friuliiensis was screened with a probe derived from the right end of the DNA insert of cosmID 18M80. Large overlapping and missing parts on cosmID 4E08 were identified. Repeated screening using a probe derived from the end of the DNA insert of the newly isolated cosmID led to the identification of cosmID SP02. The assembly of the sequences of the three overlapping cosmID inserts resulted in a stretch of approximately 70.8 kb.

The putative ORFs and genetic organization of this chromosomal region are depicted in Fig. 3. We identified putative promoters in intergenic regions with suitable sizes or with a change in the direction of transcription. We also proposed the functions of proteins encoded by the 33 detectable ORFs based on significant similarities of the deduced amino acid sequences to sequences of known proteins or conserved domains. Relevant features of the ORFs are summarized in Table 2.

Determination of the gene cluster boundaries. We could not clearly identify the boundaries of the friulimicin biosynthetic gene cluster based on the deduced functions of the gene products (Table 2). The regulatory genes regA and regD have already been shown to be a part of the gene cluster by using gene inactivation experiments (to be published elsewhere). To determine whether orfO’ and orfAP, located upstream of regA, and orfP and orfTD, located downstream of regD (Fig. 3), represent friulimicin biosynthetic genes, the corresponding genes were inactivated by gene disruption (orfO’ and orfAP) and gene replacement (orfP and orfTD) using plasmids pCMF11/pCMF10 and PCMOP/Apra, respectively (see Materials and Methods). The genotypes of the generated mutants were proven by Southern hybridization (data not shown) and analyzed in a bioassay (see Materials and Methods). Inactivation of any of these genes had no effect on friulimicin biosynthesis (data not shown), and therefore these genes are not involved. It is reasonable to assume that regA and regD represent the left and right ends of the cluster.

With this assumption, a total of 24 ORFs define the friulimicin biosynthetic gene cluster, covering approximately 61.1 kb.

NRPS. Four genes of the friulimicin biosynthetic gene cluster encode the nonribosomal peptide synthetases PstA to PstD. Similarity to PstB and PstD was found in the daptomycin...
TABLE 2. Genes in the friulimicin biosynthetic gene cluster region of *A. friuliensis*

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size (bp)</th>
<th>Protein(s), organism, and accession no. corresponding to gene with sequence similarity</th>
<th>e value</th>
<th>Proposed function</th>
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</thead>
<tbody>
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<td>orfO</td>
<td>ND</td>
<td>Choline oxidase, <em>Arthrobacter globiformis</em>, AJ304845.1</td>
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<td>Unknown</td>
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<td>729</td>
<td>Regulator BrPa, <em>Streptomyces hygroscopicus</em>, Q01108</td>
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<td>expB</td>
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<td>ABC transporter, ATP-binding cassette, <em>Streptomyces griseus</em>, AJ300320.1</td>
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<td>daB</td>
<td>1,047</td>
<td>Cysteine synthase, <em>Mesorhizobium loti</em>, NC002679.1</td>
<td>1e-76</td>
<td>2,3-Diaminobutyric acid synthesis</td>
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<tr>
<td>dafB</td>
<td>1,443</td>
<td>Fusion protein containing a putative ligase and an argininosuccinate lyase, <em>Mesorhizobium loti</em>, AL672113.1</td>
<td>1e-56</td>
<td>2,3-Diaminobutyric acid synthesis</td>
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<tr>
<td>dafC</td>
<td>1,224</td>
<td>Fusion protein containing a putative ligase and an argininosuccinate lyase, <em>Mesorhizobium loti</em>, AL672113.1</td>
<td>8e-37</td>
<td>2,3-Diaminobutyric acid synthesis</td>
</tr>
<tr>
<td>psa</td>
<td>3,189</td>
<td>Pyoverdine synthetase A, <em>Pseudomonas fluorescens</em>, AF237701.4</td>
<td>6e-93</td>
<td>Peptide synthesis</td>
</tr>
<tr>
<td>orf7</td>
<td>3,189</td>
<td>Pyoverdine synthetase A, <em>Pseudomonas fluorescens</em>, AF237701.4</td>
<td>6e-93</td>
<td>Peptide synthesis</td>
</tr>
<tr>
<td>regB</td>
<td>906</td>
<td>SrpP-like protein, <em>Streptomyces avermitilis</em>, AB070953.1</td>
<td>3e-87</td>
<td>Regulation</td>
</tr>
<tr>
<td>expA</td>
<td>840</td>
<td>ABC transporter, transmembrane component, <em>Streptomyces griseus</em>, AJ300320.1</td>
<td>5e-40</td>
<td>Transport/Resistance</td>
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<tr>
<td>glmB</td>
<td>1,248</td>
<td>NikV protein, <em>Streptomyces tendae</em>, AJ250581.1</td>
<td>5e-73</td>
<td>Methylaspartic acid synthesis</td>
</tr>
<tr>
<td>glmA</td>
<td>438</td>
<td>NikU protein, <em>Streptomyces tendae</em>, AJ250581.1</td>
<td>2e-27</td>
<td>Methylaspartic acid synthesis</td>
</tr>
<tr>
<td>lipA</td>
<td>1,767</td>
<td>Acyl-CoA ligase, <em>Actinoplanes teichomyceticus</em>, AJ605139.1</td>
<td>8e-123</td>
<td>Acyl-CoA/AMP synthesis</td>
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<tr>
<td>lipB</td>
<td>1,539</td>
<td>Acyl-CoA dehydrogenases, <em>Burkholderia mallei</em>, CP000011.2</td>
<td>6e-102</td>
<td>Formation of the Δcis3 double bond in the acyl residue</td>
</tr>
<tr>
<td>orf12</td>
<td>708</td>
<td>None</td>
<td>2e-09</td>
<td>Unknown</td>
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<td>lipD</td>
<td>267</td>
<td>Putative peptide carrier protein, <em>Streptomyces avermitilis</em>, NC003155.3</td>
<td>2e-09</td>
<td>Synthesis of the acyl residue</td>
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<tr>
<td>pstB</td>
<td>9,432</td>
<td>Peptide synthetase 1, <em>Streptomyces filamentosus</em>,&lt;sup&gt;a&lt;/sup&gt; AY877762.1</td>
<td>0.0</td>
<td>Peptide synthesis</td>
</tr>
<tr>
<td>pstC</td>
<td>19,986</td>
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<tr>
<td>pstD</td>
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<td>orf21</td>
<td>213</td>
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<td>lipE</td>
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<td>pip</td>
<td>1,020</td>
<td>Lysine cyclodeaminase, <em>Streptomyces hygroscopicus</em>, AF235504.1</td>
<td>6e-84</td>
<td>Pipelicolic acid synthesis</td>
</tr>
<tr>
<td>regC</td>
<td>645</td>
<td>Putative two-component response regulator, <em>Streptomyces coelicolor</em>, AL939112.1</td>
<td>8e-80</td>
<td>Regulation</td>
</tr>
<tr>
<td>regD</td>
<td>1,380</td>
<td>Putative two-component sensor kinase, <em>Streptomyces coelicolor</em>, AL939112.1</td>
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<td>Regulation</td>
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<tr>
<td>orfP</td>
<td>612</td>
<td>Acid phosphatase, class B, <em>Legionella pneumophila</em>, NC002942.5</td>
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<td>Unknown</td>
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<td>mem2</td>
<td>1,590</td>
<td>Antibiotic efflux protein, <em>Streptomyces atroolivaceus</em>, AF485561.1</td>
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<td>Antibiotic efflux</td>
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<tr>
<td>regE</td>
<td>624</td>
<td>Putative TetR family transcriptional regulator, <em>Streptomyces avermitilis</em>, BA000030.2</td>
<td>2e-26</td>
<td>Regulation</td>
</tr>
<tr>
<td>orfTD</td>
<td>969</td>
<td>Threonine dehydratase, <em>Burkholderia pseudomallei</em>, 1710b, NC07434.1</td>
<td>1e-67</td>
<td>Threonine degradation</td>
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<td>orf31</td>
<td>871</td>
<td>Hypothetical protein SCL6.09, <em>Streptomyces coelicolor</em>, AL939109.1</td>
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<td>Unknown</td>
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<tr>
<td>orf32</td>
<td>1,194</td>
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<tr>
<td>orf33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>None</td>
<td></td>
<td>Unknown</td>
</tr>
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</table>

<sup>a</sup> ORFs listed in bold are part of the friulimicin biosynthetic gene cluster.

<sup>b</sup> ND, not determined.

<sup>c</sup> Accession numbers are for sequences deposited in the NCBI GenBank.

<sup>d</sup> *Streptomyces filamentosus* is identical to the daptomycin producer strain (formerly called *Streptomyces roseosporus*).

<sup>e</sup> e value is defined in reference 2.

*Streptomyces filamentosus* producer genes (Table 2). The domain organization was analyzed in silico using the highly conserved core motifs of NRPS (for a review, see reference 39). According to the peptide structure of friulimicin, 11 adenylating domains were found: one in PstA, two in PstB, six in PstC, and two in PstD. The gene encoding the monomodular synthetase PstA is separated from the other NRPS genes by a set of nine genes involved in transport and regulation, the synthesis of methylaspartic acid, and the synthesis and linkage of the fatty acid component. The N-terminal region of PstA (approximately 210 amino acids) shows no sequence similarity to any NRPS or polyketide synthase domains with known functions. Epimerization domains are located at the ends of PstB and PstC in the modules 3 and 9, in accordance with the π conformation of piperolic acid and the Dab-9 amino acid residue. This result is consistent with the published stereochemical structure of the friulimicin and amphomycin peptides (7, 48). PstD ends with a thioesterase domain for regiospecific cyclization. The friulimicin branched lactam ring is built from the C terminus of proline and the amino side chain of Dab-2.
The thioesterase domain may be a useful tool for the chemoenzymatic generation of other lipopeptide derivatives. Such an approach has been used for the synthesis of daptomycin derivatives with the recombinant cyclization domain of the *Streptomyces coelicolor* CDA NRPS (12).

**The synthesis of nonproteinogenic amino acids.** The peptide core of friulimicin includes methylaspartic acid, which is provided by a glutamate mutase complex encoded by genes *glmA* and *glmB* (15), and the nonproteinogenic amino acids pipecolinic acid at position 3 and 2,3-diaminobutyric acid at positions 2 and 9 (Fig. 1).

The deduced amino acid sequence of the *pip* gene product (Pip; 339 amino acids) shows high similarities to the sequences of lysine and ornithine cyclodeaminases; the former are thought to be responsible for the synthesis of pipecolinic acid.

**rapL** in the rapamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* encodes a protein with 48% sequence identity to Pip. RapL has high sequence similarity to an ornithine cyclodeaminase from *Agrobacterium tumefaciens* (12). It catalyzes the deaminative cyclization of lysine to pipecolinic acid; therefore, the enzyme is considered to be a lysine cyclodeaminase (27).

To determine whether *pip* is involved in friulimicin biosynthesis, we inactivated the gene by replacing an internal 979-bp NcoI-XhoI fragment with an apramycin resistance cassette, resulting in the mutant AF-PIP-32. The genotype of the mutant was confirmed by Southern hybridization (data not shown). The mutant did not produce friulimicin, as indicated by the loss of antibiotic activity in the bioassay (data not shown). This phenotype was attributed to the loss of *pip* gene function by successful genetic complementation and the subsequent restoration of friulimicin production (see Materials and Methods). Mutant AF-PIP-32 had friulimicin activity when grown on M65 medium supplemented with racemic pipecolinic acid, whereas other nonproducing mutants, e.g., AF-O3N-10 and AF-O4-23 (see below), did not have antibiotic activity when grown on this medium.

To verify pipecolinic acid production by Pip, the *pip* gene on plasmid pRF37 under the control of the T7 promoter was heterologously expressed in *E. coli* BL21(DE3)pLys (see Materials and Methods). Soluble N-terminally His-tagged Pip was produced, as shown by purification using metal chelate affinity chromatography with Ni-nitrilotriacetic acid resin under native conditions and subsequent Western blot analysis using the antibody epitope (Xpress epitope) in the recombinant protein for visualization (Fig. 4A).

To show the in vivo production of pipecolinic acid, cell pellets of the expression strain *E. coli* BL21(DE3)pLys(pRF37) and of *E.
coli BL21(DE3)pLys(pRSETB) were extracted twice with 80% ethanol, and the corresponding ethanol-soluble fractions were analyzed by HPLC and TLC and compared. The HPLC profile of the E. coli BL21(DE3)pLys(pRF37) extract revealed a new peak at about 3.1 min; no such peak was observed for the negative control E. coli BL21(DE3)pLys(pRSETB) (Fig. 4C). The mass spectrometry spectrum of the new peak revealed the expected molecular mass of pipecolinic acid (Fig. 4D). The results were confirmed by coinjection with pipecolinic acid as the internal standard. With the detection of L-pipecolinic acid in the ethanol-soluble fraction of the expression strain, the in vivo activity of the His-tagged lysine cyclodeaminase equivalent in a heterologous host was demonstrated. The chiral specificity of the heterologously produced enzyme in E. coli BL21(DE3)pLys was determined by chiral-plate TLC and densitometric analysis. Only L-pipecolinic acid was found within the ethanol-soluble fraction of the expression strain (Fig. 4B).

Owing to the ability to introduce reverse turns into peptides, pipecolinic acid and its derivatives are frequently used as a structural moiety in a number of synthetic pharmaceuticals (35, 44). The incorporation of unusual amino acids such as pipecolinic acid also seems to increase the proteolytic stability of these compounds. The biological activity of a molecule is often dependent upon its absolute configuration (35). Therefore, it would be desirable to have access to nonracemic pipecolinic acid. Pip seems to be a good candidate for the biotechnological production of L-pipecolinic acid, which can then be used as a building block for the synthesis of bioactive peptides.

To date, the biosynthetic mechanism of 2,3-diaminobutyric acid is unknown. We attempted to elucidate the steps in 2,3-diaminobutyric acid synthesis by identifying genes within the friulimic biosynthetic gene cluster encoding proteins with similarities to enzymes of amino acid metabolism. The ORFs designated dabA and dabB and dabC encode proteins with similarities to cysteine synthases and to a fusion protein containing both an argininosuccinate lyase and a ligase domain, respectively (Table 2). Equivalents of these ORFs are present in the symbiosis island of Mesorhizobium loti (43), a rhizobacterium that also contains small amounts of 2,3-diaminobutyric acid (40). We inactivated dabA and dabB to test their involvement in 2,3-diaminobutyric acid synthesis. The dabA gene was subjected to insertional mutagenesis using plasmid pCMO3ApraN. This plasmid carries the apramycin/ermE*p resistance cassette aprP in the middle of the dabA gene. A dabB knockout mutant was constructed by replacing a 1,011-bp NcoI-SacI fragment of the gene with the aprP cassette by using plasmid pCMO4Apra. Since in silico data suggest that the genes expB, dabA, dabB, dabC, pstA, and orf7 are translationally coupled, they may together form an operon. Therefore, ermE*p was introduced in the transcriptional direction of the
inactivated genes in both mutants to lessen the polar effects on genes downstream of the inactivation site. The genotypes of the resulting apramycin-resistant, kanamycin-sensitive mutants AF-O3N-10 and AF-O4-23 were confirmed by Southern hybridization (Fig. 5A to D). In these experiments, instead of a 4.6-kb BamHI fragment characteristic of the wild-type DNA, hybridizing 1.7-kb and 0.9-kb BamHI fragments in the corresponding dabA and dabB mutants, respectively, were detected. Friulimicin production was abolished in both mutants (Fig. 5E), which indicated that both genes are essential for friulimicin biosynthesis. The mutants were genetically complemented by the cloning of a native 6,651-bp StuI-Eco47III fragment of cosmid 18M80, carrying part of the fimE*p (AF-O3-10) and not to the dabB knockout mutant. These results indicated that the dabB mutant is blocked at a later step of the reaction than the dabA mutant.

Regarding the in silico data and the experimental results for dabA and dabB, one can conclude that DabA, DabB, and DabC are involved in the synthesis of 2,3-diaminobutyric acid. Since DabA has similarity to the pyridoxal phosphate-dependent cysteine synthases, a β replacement or elimination reaction may be engaged. For the synthesis mechanism of the structurally related compound 2,3-diaminopropionate in the viomycin biosynthetic pathway, the role of the cysteine synthase VioB in β-substituent replacement on O-acetyl-serine has been proposed previously (46). But the mechanism for 2,3-diaminobutyric acid synthesis must still be investigated.

Regulation, transport, resistance, synthesis, and linkage of the fatty acid component. At least four genes of the friulimicin biosynthetic gene cluster show similarity to regulatory genes. RegC and RegD probably represent a two-component response regulatory system. RegA has similarity to the transcriptional activators of bialaphos biosynthesis, BrpA from Streptomyces hygroscopicus and PrpA from Streptomyces viridochromogenes (31, 38). RegB is a SyrP-like protein (54) and may be involved in a phosphorylation cascade.

expA and expB encode the two components of an ABC transporter. The genes encoding the two components of other ABC transporters in antibiotic-producing actinomycetes lie next to each other (23). The expA and expB genes of A. friuliensis, in contrast, are separated by a number of other ORFs and are transcribed in opposite directions. The mem gene possibly encodes an integral membrane protein that functions in friulimicin transport.

Friulimicin probably inhibits peptidoglycan synthesis by the complexing of the carrier undecaprenyl monophosphate in analogy to the mode of action of the structurally related antibiotic amphomycin (5, 45). Since the target lies inside the cell, antibiotic efflux may be an important mechanism of self-resistance. We provided evidence for such a self-resistance mechanism involving expA, which encodes the transmembrane component of an ABC transporter. The expA gene under the control of the constitutive promoter ermE*p on plasmid pEM4ExpA was overexpressed in the friulimicin-sensitive strain Streptomyces lividans TK23. The MIC for Streptomyces lividans TK23 transformed with the vector pEM4 as a control was 1 to 5 μg ml−1. In contrast, the MIC for Streptomyces lividans TK23 transformed with pEM4ExpA was 50 to 75 μg ml−1. The increase in friulimicin resistance owing to expA expression was observed even though the ATP-binding domain of the corresponding ABC transporter was missing in the heterologous host. This means that ExpA must have nonspecifically interacted with another ATP-binding domain produced by Streptomyces lividans.

The genes lipA, lipB, and lipD are likely to have a role in the synthesis and linkage of the fatty acid component of friulimicin. The deduced product of lipA has similarity to acyl coenzyme A (acyl-CoA) ligases, exhibits the conserved AMP-binding domain motif, and therefore probably represents an acyl-AMP ligase. LipD encodes an acyl carrier protein. Equivalents of lipA and lipD are present in the gene cluster for the lipopeptide daptomycin (26). In A54145 biosynthesis, these functions are fused by the fusion of the corresponding genes in the cluster (24).
The involvement of the lipB gene product, an acyl-CoA dehydrogenase, in the formation of the Δc13 Δs2 double bond in the Δc13 Δs2 residue has previously been shown by insertional inactivation and heterologous expression of the gene (14). lipE encodes a protein belonging to the β,β-hydrolase family, which includes acyltransferases and thioesterases. Whether this enzyme is involved in an acyltransferase reaction, as predicted in the acylation model of friulimicin biosynthesis (14), is not clear. A thioesterase type II function for the regeneration of misprimed NRPS is also possible (39). To date, an acyltransferase involved in such an antibiotic biosynthetic pathway has been found only for teicoplanin biosynthesis in Actinoplanes telicromyceticus, where it acylates the sugar moiety (22). Acyltransferase genes have not been identified in other biosynthetic gene clusters, especially those for lipopeptides such as CDA, daptomycin, and surfactin (17, 26, 42).

The products of orf7 (putative kinase), orf12 (hypothetical protein), and orf21 (MBT-like family protein) have unknown functions. Among these genes, orf21 represents a highly conserved gene with equivalents in many gene clusters corresponding to nonribosomally synthesized peptides (e.g., references 8, 26, 38, 41, 51, and 53).

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REFERENCES


