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# An acyl-CoA dehydrogenase is involved in the formation of the Δ*cis*3 double bond in the acyl residue of the lipopeptide antibiotic friulimicin in *Actinoplanes friuliensis*

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The lipopeptide antibiotic friulimicin, produced by *Actinoplanes friuliensis*, is an effective drug against Gram-positive bacteria, such as methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus aureus* strains. Friulimicin consists of a cyclic peptide core of ten amino acids and an acyl residue linked to an exocyclic amino acid. The acyl residue is essential for antibiotic activity, varies in length from C13 to C15, and carries a characteristic double bond at position Δ*cis*3. Sequencing of a DNA fragment adjacent to a previously described fragment encoding some of the friulimicin biosynthetic genes revealed several genes whose gene products resemble enzymes of lipid metabolism. One of these genes, *lipB*, encodes an acyl-CoA dehydrogenase homologue. To elucidate the function of the LipB protein, a *lipB* insertion mutant was generated and the friulimicin derivative (FR242) produced by the mutant was purified. FR242 had antibiotic activity lower than friulimicin in a bioassay. Gas chromatography showed that the acyl residue of wild-type friulimicin contains a double bond, whereas a saturated bond was present in FR242. These results were confirmed by the heterologous expression of *lipB* in *Streptomyces lividans* T7, which led to the production of unsaturated fatty acids not found in the *S. lividans* T7 parent strain. These results indicate that the acyl-CoA dehydrogenase LipB is involved in the introduction of the unusual Δ*cis*3 double bond into the acyl residue of friulimicin.

## INTRODUCTION

In recent years, the necessity for new effective antibiotics against pathogenic organisms has attained increasing significance in clinical treatment because of bacterial resistance to frontline antibiotics, such as $\beta$-lactam, aminoglycoside and glycopeptide antibiotics. In certain cases, not even the glycopeptide antibiotic of last resort, vancomycin, is effective against the methicillin-resistant Gram-positive *Enterococcus* and *Staphylococcus* strains (Moellering, 1998; Barie, 1998). Therefore, new effective antibiotics against multidrug-resistant infectious bacteria are essential to treat the diseases they cause.

Lipopeptide antibiotics are one class of antibiotics effective against Gram-positive bacteria, and with Cubicin (daptomycin), a first member of this class was approved by the US Food and Drug Administration in 2003.

Eight lipopeptides recently identified and isolated from the actinomycete *Actinoplanes friuliensis* (Aretz *et al.*, 2000) are composed of an identical cyclic peptide structure of ten amino acids. The peptide core is N-terminally linked via diaminobutyric acid to an acylated, exocyclic amino acid, either asparagine or aspartate (Fig. 1a) (Vértesy *et al.*, 2000). The acyl residue includes branched-chain (iso and anteiso) fatty acids with a chain length of C13 to C15, all of which have an unusual double bond at position Δ*cis*3 (Fig. 1b). Four of the eight lipopeptides (A1437 A, A1437 B, A1437 E, A1437 G) have aspartate as the exocyclic amino acid and are identical to known peptide antibiotics of the amphomycin group; the other four lipopeptide structures (friulimicin A–D) have asparagine as the exocyclic amino acid and represent a new class of antibiotics (Vértesy *et al.*, 2000) (Fig. 1b).

Abbreviations: ACP, acyl carrier protein; CDA, calcium-dependent antibiotic.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the friulimicin biosynthetic genes reported in this paper is AJ488769.



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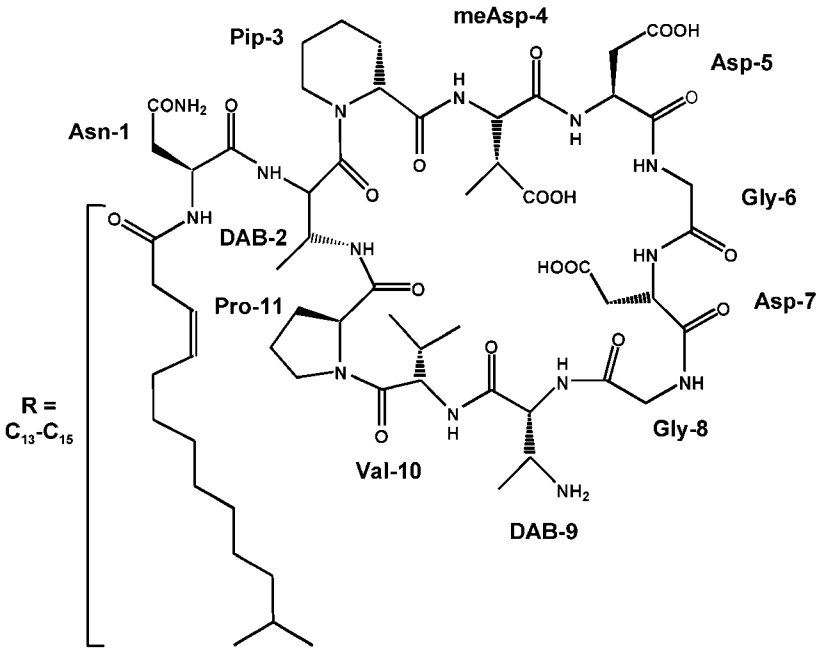
(a)




(b)

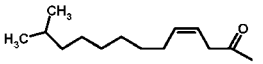
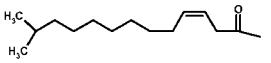
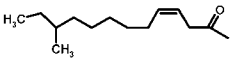
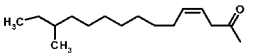
| Lipopeptides | aa Residue at pos. 1 | Fatty acid part (=R) |
|---|---|---|
| Friulimicin A | Asn |  |
| A1437 A | Asp | iso-C13 |
| Friulimicin B | Asn |  |
| A1437 B | Asp | iso-C14 |
| Friulimicin C | Asn |  |
| A1437 E | Asp | anteiso-C13 |
| Friulimicin D | Asn |  |
| A1437 D | Asp | anteiso-C15 |

**Fig. 1.** Chemical structures of the lipopeptide antibiotics from *Actinoplanes friuliensis*. (a) Chemical structure of friulimicin B. The amino acid positions are indicated by numbers. DAB, diaminobutyrate; Pip, pipecolate; Asn, asparagine; Asp, aspartate; Gly, glycine; Val, valine; Pro, proline; meAsp, methylaspartate. (b) Exocyclic amino acid at position 1 and fatty acid moiety (R) of the eight lipopeptide antibiotics.



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The synthesis of the peptide core, which carries amino acids with a partially aproteinogenic character, such as methylaspartate and pipecolate, has been postulated to occur non-ribosomally via peptide synthases. By reverse genetics, a 9·2 kb portion of the friulimicin biosynthetic gene cluster, which carries genes encoding glutamate mutase and proteins involved in the non-ribosomal synthesis, has been identified (Heinzelmann *et al.*, 2003) (Fig. 2).

The target of lipopeptide antibiotics is in almost all cases cell wall biosynthesis, resulting in the disruption of ion transport processes and the electric potentials of biological membranes (Steller *et al.*, 1999). Specific inhibition mechanisms have also been described; for example, ramoplanin produced by *Actinoplanes* sp. strain ATCC 33076 arrests cell wall biosynthesis at a late stage of peptidoglycan biosynthesis (Hu *et al.*, 2003). A correlation between the bactericidal activity of daptomycin and membrane depolarization has been shown (Silverman *et al.*, 2003). For many other lipopeptide antibiotics, including friulimicin, the calcium-dependent antibiotic (CDA) and amphomycins, a similar mode of action has been postulated (Hojati *et al.*, 2002). Friulimicin seems to inhibit cell wall biosynthesis by complexing the C55-isoprenoid lipid carrier bactoprenylphosphate (H. Decker, Frankfurt, personal communication).

The clinical application of these antibiotics is often limited by their toxicity to eukaryotic cells. For example, friulimicin displays haemolytic activity against erythroplasts (H. Decker, Frankfurt, personal communication). The action and also the toxicity of lipopeptide antibiotics strongly depend on the moiety of the acyl residue. Therefore, the synthesis of lipopeptide antibiotics consisting of known peptide structures and a different acyl residue could lead to new antibiotics active against a wide range of bacteria, but with fewer side effects.

The biosynthesis of the peptide moiety of lipopeptide antibiotics via non-ribosomal peptide synthases has been well characterized; however, less is known about the synthesis of the acyl residue and its linkage to the peptide moiety. Several lipopeptide antibiotic biosynthetic gene clusters have been isolated and partially characterized, such as fengycin (Tosato *et al.*, 1997), mycosubtilin (Duitman *et al.*, 1999), lichenysin (Konz *et al.*, 1999), syringomycin (Guenzi *et al.*, 1998), daptomycin (McHenney *et al.*, 1998) and CDA (Chong *et al.*, 1998; Hojati *et al.*, 2002; Ryding *et al.*, 2002). In addition to peptide synthase genes, genes probably involved in the synthesis of the lipid moiety of the antibiotics have been found in the gene clusters.

The synthesis of the 2,3-epoxyhexanoyl acyl residue of the CDA from *Streptomyces coelicolor* A3(2) has been postulated by Hojati *et al.* (2002) to involve primary and secondary metabolic enzymes. In their model, the precursor hexanoyl-acyl carrier protein (ACP) is synthesized by fatty

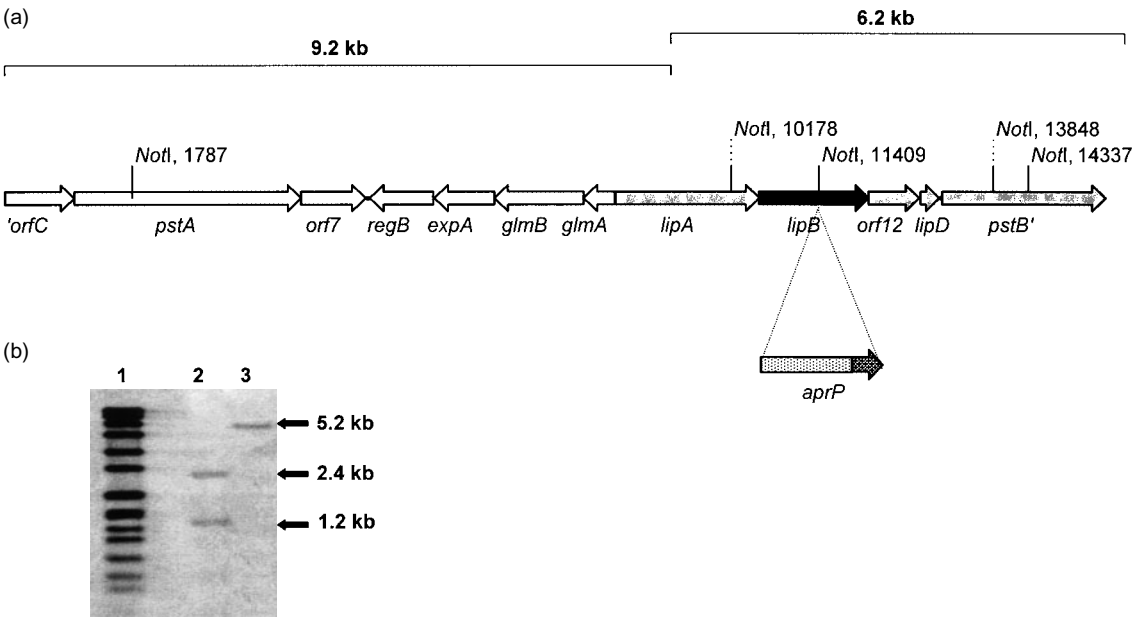


**Fig. 2.** Genetic localization and insertion mutagenesis of the *lipB* gene. (a) Arrangement of the genes on a 15·4 kb DNA fragment of the friulimicin biosynthetic gene cluster. The 9·2 kb fragment determined previously (Heinzelmann *et al.*, 2003) and the 6·2 kb region described here are shown. The insertion of the resistance gene cassette *aprP* within the *lipB* gene in the mutant MEHB is indicated. (b) The correct gene insertion mutagenesis in mutant MEHB was verified by Southern hybridization using the *lipB* gene as probe. Lanes: 1, DNA molecular size markers VII, DIG-labelled (Roche); 2, *Not*I-digested chromosomal DNA of *A. friuliensis* wild-type; 3, *Not*I-digested chromosomal DNA of mutant MEHB. The hybridizing *Not*I fragments are marked by arrows.


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acid synthases, enzymes of primary metabolism. Hexanoyl-ACP is then hydrolysed by a thioesterase, and free hexanoyl is activated by acyl-CoA synthase to form hexanoyl-CoA. These two enzymes might also be recruited from primary metabolism, since neither a gene encoding a thioesterase nor a gene encoding an acyl-CoA synthase has been found within the complete CDA biosynthetic gene cluster. In contrast, the subsequent two reactions responsible for the formation of the 2,3-epoxyhexanoyl group are carried out by two proteins of secondary metabolism, an oxidase and a monooxygenase, encoded by the genes *hxcO* and *hcmO*, both located within the CDA gene cluster. The peptide synthase CdaPS1, which activates the first amino acid, is postulated to transfer and attach 2,3-epoxyhexanoyl to the first amino acid (Hojati *et al.*, 2002).

Another example of an acylation mechanism in the synthesis of peptide structures has been described for mycosubtilin biosynthesis in *Bacillus subtilis* ATCC 6633 (Duitman *et al.*, 1999). Here, a multifunctional hybrid of a peptide synthase, an amino transferase and a fatty acid synthase are responsible for the synthesis of mycosubtilin. In the hypothetical model of synthesis of the lipid moiety, a long-chain fatty acid is activated by an acyl-CoA synthase domain. In the next step, the activated acyl residue binds the 4′-phosphopantetheine cofactor of an ACP domain. Malonyl-CoA is loaded onto a second ACP domain, and the formation of a $\beta$-ketoacylthioester is catalysed by a $\beta$-ketoacylsynthase domain. After amination of the keto group, the lipid moiety is attached to the first amino acid of the peptide by the condensation domain of the peptide synthase (Duitman *et al.*, 1999).

To elucidate the acylation mechanism of friulimicin, we isolated a 6·2 kb DNA fragment flanking the previously isolated 9·2 kb portion of the friulimicin biosynthetic gene cluster (Heinzelmann *et al.*, 2003) and identified genes belonging to this cluster that are involved in the synthesis of the acyl residue. We demonstrated the involvement of a potential FAD-dependent acyl-CoA dehydrogenase, encoded by one of these genes (*lipB*), in the introduction of the unusual $\Delta$*cis*3 double bond within the acyl residue.

## METHODS

**Cloning, restriction mapping and *in vitro* manipulation of DNA.** Methods for the isolation and manipulation of DNA were as described by Sambrook *et al.* (1989) and Hopwood *et al.* (1985). Restriction endonucleases were used according to the manufacturer's instructions.

**Intergeneric conjugation between *Escherichia coli* and *A. friuliensis*.** Intergeneric conjugation followed the procedure described by Heinzelmann *et al.* (2003).

**Gene disruption, mutagenesis and transformation.** A gene disruption mutant was generated by intergeneric conjugation between *A. friuliensis* and *E. coli* ET12567/pUB307 (Heinzelmann *et al.*, 2003) with plasmid pEHLBA2 (Table 1). *Streptomyces lividans* T7 (Fischer, 1996) protoplasts were transformed using PEG, as described by Hopwood *et al.* (1985). *E. coli* was transformed using the $CaCl_2$ method described by Sambrook *et al.* (1989). For standard cloning experiments, *E. coli* XL-1 Blue was used.

**Southern hybridization.** Southern hybridization was performed using the non-radioactive DIG DNA Labelling and Detection Kit from Roche. Hybridization using the *lipB* gene as a probe was carried out at 68 °C with a stringent washing step with 0·5× SSC/0·1 % SDS.

**DNA sequencing and analysis.** DNA fragments of the friulimicin biosynthetic gene cluster were subcloned in the sequencing vectors pUC18 and pK18 or pK19. The nucleotide sequences were determined by standard techniques (Sanger *et al.*, 1977). The codon usage programme of Staden & McLachlan (1982) was used to identify ORFs. Homology searches and multiple sequence alignments were performed using the programs BLAST (Altschul *et al.*, 1990), CLUSTAL W (Thompson *et al.*, 1994) and Genedoc (Nicholas *et al.*, 1997).

**Isolation of *lipB*.** For the construction of the *lipB* mutant MEHB and the overexpression strain *S. lividans* T7/pEHEX3, an internal *lipB* fragment (*lipB**) and the complete *lipB* gene, respectively, each carrying a *Bgl*II and *Hin*dIII restriction site at the 5′ and 3′ termini, were isolated using PCR. Plasmid pOP2, which contains a 5·6 kb *Pst*I fragment of the cluster carrying the 1·5 kb *lipB* gene, was used as template. The following reaction mixtures were used: 0·5 µg pOP2; primer M1 (5′-AAT<u>AGATCT</u>GCGAAGCTCGACGAGG-CCGAG-3′) and primer M2 (5′-AAT<u>AAGCTT</u>CTCCAGCAAC-GCCACGAGACG-3′) for the isolation of *lipB** or primer E1 (5′-AAT<u>AGATCT</u>ATGACGGACCTGTCCACCCTG-3′) and primer E2 (5′-AAT<u>AAGCTT</u>TCATCGGGCACCGACCCCG-3′) for the isolation of *lipB* (sequences of restriction sites used for cloning are underlined; 100 pmol each); 10 µl of 10× reaction buffer (containing 20 mM $MgCl_2$); 5 % DMSO; 0·2 mM deoxynucleoside triphosphates; and 1 U of *Pwo* polymerase (Roche). After a denaturation step (5 min, 98 °C), 25 cycles of amplification (1 min 94 °C, 1 min 69 °C, 1·5 min 72 °C) were performed. The 1·4 kb *lipB** and the 1·5 kb *lipB* PCR products were electrophoretically separated in a 1 % agarose gel, eluted from the gel, and cloned into the vector pJOE890, resulting in plasmids pEHLB1 and pEHEX1, respectively (Table 1).

**Construction of plasmids pEHLBA2 and pEHEX3.** For the construction of the *lipB* mutant, *lipB** was isolated from pEHLB1 as a *Bgl*II–*Hin*dIII fragment and cloned into *Bam*HI–*Hin*dIII-digested vector pK19, resulting in pEHLB2 (Table 1). pEHLB2 was digested with *Not*I, whose recognition sequence is located approximately in the middle of *lipB**, and the resulting overhanging ends were filled in with Klenow polymerase. The apramycin/PermE resistance cassette *aprP* was isolated by a *Stu*I–*Eco*RV restriction of plasmid pEH13 (Heinzelmann *et al.*, 2001). To lessen possible polar effects, *aprP* was inserted in the transcriptional direction of *lipB** (*lipBaprP*) in plasmid pEHLB2, resulting in plasmid pEHLBA1 (Table 1). A 3 kb *Kpn*I–*Hin*dIII fragment of pEHLBA1 carrying *lipBaprP* was cloned in the *Kpn*I–*Hin*dIII-digested vector pK18mob (Schäfer *et al.*, 1990). The resulting plasmid pEHLBA2 (Table 1) was transferred to *A. friuliensis* by intergeneric conjugation between *E. coli* 12567/pUB307 and *A. friuliensis*, as described by Heinzelmann *et al.* (2003).

For the expression of *lipB* in *S. lividans* T7, the *lipB* gene was isolated as a *Bgl*II–*Hin*dIII fragment from pEHEX1 and cloned in *Bgl*II–*Hin*dIII-restricted pRSETB, resulting in plasmid pEHEX2 (Table 1). pEHEX2 was cloned as a *Hin*dIII fragment into vector pGM9, resulting in the *Streptomyces*–*E. coli* shuttle plasmid pEHEX3 (Table 1).

**Bioassay for antibiotic production.** The antibiotic production of the mutant MEHB and of the wild-type was tested in a bioassay


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**Table 1.** Bacterial strains and plasmids used in this study

| Strains and plasmids | Relevant genotype and phenotype | Source or reference |
|---|---|---|
| ***A. friuliensis*** | | |
| HAG010964 | Friulimicin-producing wild-type | Aretz *et al.* (2000) |
| MEHB | Non-friulimicin-producing mutant, apra$^r$ | This study |
| ***S. lividans*** | | |
| T7 | *tsr*, T7 RNA polymerase gene | Fischer (1996) |
| T7/pEHK | *S. lividans* T7 carrying pEHK | Heinzelmann *et al.* (2003) |
| ***E. coli*** | | |
| ET12567/ pUB307 | F$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR lacYI*, pUB307 | Flett *et al.* (1997) |
| XL-1 Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB*, *lacI*$^q$*Z*Δ M15Tn*10* tet$^r$] | Bullock *et al.* (1987) |
| **Plasmids** | | |
| pK18mob | pK18 derivative carrying the *oriT* region of plasmid RP4, kan$^r$ | Schäfer *et al.* (1990) |
| pOP2 | pK19 carrying a 5·6 kb *Pst*I fragment of the friulimicin biosynthetic gene cluster | Heinzelmann *et al.* (2003) |
| pRSETB | T7-promoter expression vector, 6 × His tag, *bla* | Invitrogen |
| pGM9 | *aphII*, *ble*, *tsr*, temperature-sensitive *Streptomyces* vector | Muth *et al.* (1989) |
| pEH15 | pK19 carrying the *ermE* promoter (PermE) as a 0·3 kb *Bam*HI–*Kpn*I fragment | Heinzelmann *et al.* (2001) |
| pEHK | pGM9 carrying pRSETB as *Hin*dIII fragment | Heinzelmann *et al.* (2003) |
| pJOE890 | *bla*, 2 multiple cloning sites, 2 *ter* | Altenbuchner *et al.* (1992) |
| pEHLB1 | pJOE890 carrying *lipB* as a PCR-generated fragment | This study |
| pEHLB2 | pK19 carrying *lipB* as *Bgl*II–*Hin*dIII fragment of pEHLB1 | This study |
| pEHLBA1 | pEHLB2 carrying *aprP* as *Eco*RV–*Stu*I fragment of pEH15 in the same direction as the *lipB* fragment | This study |
| pEHLBA2 | pK18mob carrying *lipBaprP* as *Kpn*I–*Hin*dIII fragment of pEHLBA1 | This study |
| pEHEX1 | pJOE890 carrying *lipB* as a PCR-generated fragment | This study |
| pEHEX2 | pRSETB carrying *lipB* as *Bgl*II–*Hin*dIII fragment in its *Bam*HI–*Hin*dIII sites | This study |
| pEHEX3 | pGM9 carrying pEHEX2 as *Hin*dIII fragment | This study |

using *B. subtilis* as the test organism. The gene insertion mutant MEHB and the wild-type were each cultivated in M65 medium (Heinzelmann *et al.*, 2003) for 7 days. The mutant and the wild-type grew at the same rate and to the same extent. Culture broth containing mycelium pellets was homogenized and the $OD_{600}$ was determined. Both MEHB and the wild-type reached an $OD_{600}$ of approx. 2·7. Ten millilitres of each culture was centrifuged and each cell pellet was spread uniformly on the surface of a defined M65 agar plate (35 ml per plate, plate diameter 9 cm). After 7 days of incubation at 30 °C, agar plugs of equal size were cut out and applied to the *B. subtilis* test medium according to the method of Alijah *et al.* (1991). The plates were incubated overnight at 37 °C and antibiotic production was detected by the formation of a zone of growth inhibition around the agar blocks.

**Purification of friulimicin and the friulimicin derivative FR242 from culture supernatants.** For purification of friulimicin and the friulimicin derivative FR242 from culture supernatants, the wild-type and mutant MEHB were each cultivated in 100 ml seed medium (Aretz *et al.*, 2000) in a 500 ml Erlenmeyer flask for 5 days at 30 °C on a rotary shaker at 180 r.p.m. Antibiotic production medium (Aretz *et al.*, 2000; 2 × 500 ml in 1 l Erlenmeyer flasks) was inoculated with 5 ml of culture grown on seed medium and cultivated for 7 days at 30 °C at 180 r.p.m. Cells were removed by centrifugation at 5000 ***g*** and the supernatant was loaded onto a column packed with 100 ml degassed XAD-16 resin (Rohm and Haas) and equilibrated with 10 mM potassium phosphate buffer (pH 7·2). The column was washed and eluted as described by Vértesy *et al.* (2000). Fractions of 15 ml were collected, and the friulimicin content was determined by HPLC. The fractions containing native friulimicin or FR242 were pooled and concentrated to dryness using a rotary evaporator. The pellets were solubilized in methanol and analysed by GC and GC-MS.

**HPLC analysis of friulimicin and FR242.** Friulimicin and FR242 were analysed by reverse-phase HPLC using a steel column (125 mm × 4·6 mm) and a precolumn (20 mm × 4·6 mm) packed with Nucleosil 100C-18·5 μm (Maisch) as the stationary phase. The mobile phase was 0·1 % phosphoric acid (A) and acetonitrile (B) [linear gradient: 0–15 min, (A) 100–0 % and (B) 0–100 %; 1 min, (A) 0 %, (B) 100 %]. The antibiotics were detected using a diode array detection system at various wavelengths (210, 230, 260, 280, 310, 360 and 435 nm) at a flow rate of 2 ml $min^{-1}$ and an injection volume of 10 μl.

**Isolation of the fatty acid moiety of friulimicin and FR242.** To separate the acyl residue from the peptide core, purified friulimicin and FR242 were saponified by alkaline hydrolysis. Reagent I (1 ml; 22·5 g NaOH, 75 ml methanol, 75 ml $H_2O$) was mixed with the samples and the reaction mixture was incubated at 100 °C for 35 min. After cooling, the free fatty acids were esterified by adding 10 ml of reagent II (162·5 ml 6 M HCl, 137·5 ml methanol) and incubated for 12 min at 80 °C. Fatty acid methyl esters were



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extracted with 1·25 ml hexane with shaking for 10 min. To the organic phase, 3 ml 3 M NaOH was added, and the upper phase was used for GC and GC-MS.

**GC and GC-MS analyses of the acyl residue of friulimicin and FR242.** The acyl residues were analysed by GC (injector and flame-ionization detector 250 °C, split 1 : 10, injection volume 10 µl) using a polar capillary column [Carbowax 20-M (PEG), length 30 m, i.d. 0·32 mm, layer thickness 0·25 µm; JW Scientific] and the following temperature programme: 160–200 °C at 4 °C $min^{-1}$, 200–240 °C at 8 °C $min^{-1}$ and 240 °C for 5 min. Fatty acids were analysed by GC-MS on a non-polar capillary column (Optima 5, methylsilicone phase, length 15 m, i.d. 0·25 mm, layer thickness 0·25 µm; Macherey-Nagel) under the following conditions: temperature gradient (3 s at 90 °C, 90–200 °C at 26 °C $min^{-1}$, 200–300 °C at 8 °C $min^{-1}$ and 10 min at 300 °C); injector temperature, 230 °C; interface temperature, 220 °C; column pressure, 4 kPa; split, 1 : 4; injection volume, 1 µl; flow rate, 0·8 ml $min^{-1}$; total flow rate, 4 ml $min^{-1}$. The data were analysed using the GC-17A program (Shimadzu) and BenchTop-PBM (Palisade Corporation). Fatty acids were identified by comparison to the fatty acid standard NHI-D-Mix (Supelco).

**Hydrogenation of the acyl residue of friulimicin.** Friulimicin (5 mg) was dissolved in 0·5 ml methanol. After the addition of a spatula tip of palladium-activated carbon, molecular hydrogen was slowly added for 5 min. The reaction mixture was then centrifuged for 5 min at 15 000 ***g*** and the supernatant was placed in a 10 ml glass cup and dried under a stream of nitrogen gas. The hydrogenated friulimicin was dissolved in 500 µl hexane and used for GC and GC-MS analyses and bioassay.

**Heterologous expression of *lipB* in *S. lividans* and purification of His-tagged LipB.** The recombinant *lipB* gene (*hislipB*) cloned on plasmid pEHEX2 was expressed in *S. lividans* T7, which carries a thiostrepton-inducible T7 RNA polymerase gene (Fischer, 1996) and the His-tagged LipB protein was purified following the procedures described by Heinzelmann *et al.* (2001). For the GC-MS analysis of the recombinant *S. lividans* T7/pEHEX3 and *S. lividans* T7/pEHK (control) (Heinzelmann *et al.*, 2003), cells were grown in TSB medium (Bacto tryptic soy broth; Becton-Dickinson) instead of YEME medium.

**Determination of acyl-CoA dehydrogenase activity.** Acyl-CoA dehydrogenase activity was assayed spectrophotometrically using a standard acyl-CoA dehydrogenase assay described by Kieweg *et al.* (1997) and Lea *et al.* (2000), which is based on the reduction of FAD to $FADH_2$ during the dehydrogenase reaction. In this reaction, the blue dye 2,3-dichlorophenol indophenol ($Cl_2PIP$), as final electron acceptor, is reduced and becomes colourless, which can be followed spectroscopically at 600 nm. Commercially available CoA-activated straight-chain fatty acids (nC14–nC16; Sigma), were used as substrates.

**Nucleotide sequences and accession numbers.** The nucleotide sequence of the friulimicin biosynthetic genes reported in this paper has been assigned accession no. AJ488769 at EMBL.

## RESULTS

### Identification of three fatty acid biosynthetic genes within the friulimicin biosynthetic gene cluster

The incomplete acyl-CoA synthase gene *lipA′* is found at the right boundary of the previously described 9·2 kb segment of the friulimicin biosynthetic gene cluster (Heinzelmann *et al.*, 2003). *lipA* is postulated to participate in the synthesis of the acyl residue of friulimicin (Heinzelmann *et al.*, 2003). In order to analyse this aspect of friulimicin biosynthesis, the flanking region of 6·207 kb was sequenced to complete the *lipA* gene sequence and to identify further genes involved in the biosynthesis of the acyl residue. In addition to the missing 3′ end of *lipA*, three complete ORFs and one incomplete ORF, all transcribed in the same direction, were found (Fig. 2). The *lipA* gene and the following two ORFs (*lipB*, *orf12*) are probably translationally coupled, since their ends overlap by four nucleotides (ATGA). The complete *lipA* gene of 1767 bp encodes a protein of 588 aa that shows 34 % identity to an acyl-CoA synthase of *Mycobacterium leprae* (Cole *et al.*, 2001). The deduced amino acid sequence of 512 aa of the next ORF, *lipB*, is similar to acyl-CoA dehydrogenases from *Streptomyces avermitilis* and *Mycobacterium tuberculosis* (30 and 26 % identity, respectively; Omura *et al.*, 2001; Cole *et al.*, 1998). Downstream of *lipB*, an ORF (*orf12*) of 708 bp is found, whose gene product is similar only to hypothetical proteins of unknown function. The next ORF, *lipD*, encodes a protein of 88 aa with a theoretical molecular mass of 8·9 kDa that shows similarity to ACP domains of several putative polyketide synthases (PKS), such as PKS of *S. avermitilis* (35 % identity; Omura *et al.*, 2001) and of *S. coelicolor* A3(2) (36 % identity; Bentley *et al.*, 2002). At amino acid position 45 of LipD, a conserved serine residue was identified that might be the binding site of the 4′-phosphopantetheine arm (Diekmann *et al.*, 1997). The gene product of the last, incomplete ORF (*pstB′*) is similar to the CDA-peptide synthase I from *S. coelicolor* A3(2) (37 % identity; Bentley *et al.*, 2002) and to the actinomycin synthase from *Streptomyces chrysomallus* (37 % identity; Schauwecker *et al.*, 1998). In contrast to the peptide synthase PstA, which probably activates the first amino acid of friulimicin (Heinzelmann *et al.*, 2003), the PstB protein possesses a condensation domain and might be responsible for the elongation of the peptide core. At amino acid position 139 of PstB, a His-containing motif (HHAVLDGF) is found, which is characteristic for condensation domains.

### Gene disruption mutagenesis of the acyl-CoA dehydrogenase gene (*lipB*)

To determine whether the *lipB* gene product is involved in the biosynthesis of the acyl residue, and specifically in the introduction of the unusual Δ*cis*3 double bond, the *lipB* gene was subjected to insertion mutagenesis using plasmid pEHLBA2 following a protocol described by Heinzelmann *et al.* (2003). pEHLBA2 carries an apramycin/PermE resistance cassette *aprP* in the middle of the *lipB* fragment. This disrupted fragment is designated *lipB**. Apramycin-resistant, kanamycin-sensitive transformants of *A. friuliensis* were analysed by Southern hybridization, and clones showing a double-crossover event between the chromosomal copy of *lipB* and the mutated *lipB** located on pEHLBA2 were identified (Fig. 2b). The generated mutant, MEHB, was analysed for antibiotic production using a *B. subtilis* bioassay. The zone of growth inhibition around mutant


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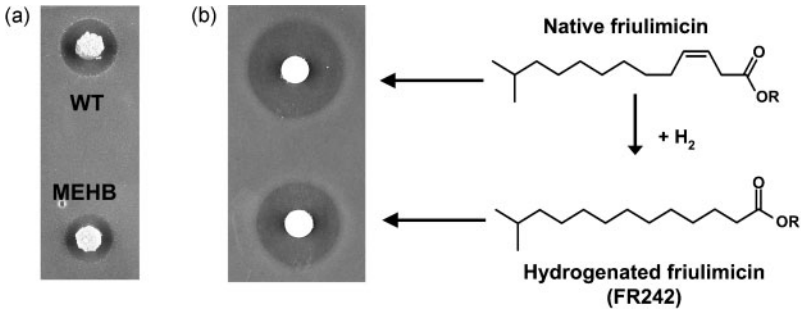


**Fig. 3.** Bioactivity of friulimicin derivatives. (a) Agar plugs with cells of *A. friuliensis* wild-type (WT) and the *lipB* mutant (MEHB) were placed on antibiotic test medium. (b) Native friulimicin (0·5 μg) and the catalytically hydrogenated friulimicin derivative FR242 (0·5 μg) were spotted onto paper discs, which were then placed on antibiotic test medium. (a) and (b) *B. subtilis* was used as the friulimicin-sensitive indicator strain. This experiment was repeated several times, and the same result was always observed.

MEHB was reproducibly smaller than that around the wild-type (approx. 62 % of the wild-type inhibition zone) (Fig. 3a), which suggested either a reduced friulimicin production or that the mutant produced a modified, less biologically active friulimicin.

## Characterization of the bioactive intermediate of mutant MEHB

In order to verify the production of a modified friulimicin by mutant MEHB, the mutant and the wild-type were each cultivated under friulimicin production conditions (Vértesy *et al.*, 2000). The culture supernatants were analysed by HPLC. The retention time of the friulimicin derivative of mutant MEHB (called FR242) did not differ significantly from that of the friulimicin standard (purified friulimicin B) and the native friulimicin of the wild-type supernatant (data not shown). Therefore, we speculated that either FR242 was unmodified or there were only slight changes in the acyl residue that were not detectable by HPLC. In order to characterize the acyl residue, friulimicin and FR242 were purified from culture supernatants by the standard friulimicin purification method (Vértesy *et al.*, 2000). The friulimicin- and the FR242-containing fractions were each pooled and concentrated to dryness. After solubilization in methanol, the acyl residues were cleaved from the peptide cores by alkaline hydrolysis, and the free acyl residues were esterified as methyl esters and then extracted with hexane. GC analysis on a polar column demonstrated the differences between FR242 and native friulimicin. Acyl residues with a chain length of iC14 (isoC14) were detected as the main component of both FR242 and friulimicin, which is in accordance with data showing that the chain length of the fatty acid moiety of friulimicin varies from C13 to C15, with C14 as the main component (H. Decker, Frankfurt, personal communication). However, the fatty acid of native friulimicin eluted with a retention time of 2·3 min, whereas the retention time of the FR242 fatty acid was 2·0 min (data not shown). Therefore, the iC14 fatty acid of FR242 seems to be less polar than that of friulimicin. Such an effect might originate from the lack of the double bond, which would give a polar character to the fatty acid.

To analyse whether the difference in the retention times can really be attributed to the presence or absence of an unsaturated bond, the masses were determined by GC-MS. An overlay of the iC14 : 0 (from FR242) and iC14 : 1 (from friulimicin) spectra demonstrated the different retention times (Fig. 4a). Owing to the non-polar character of the column used, iC14 : 1 elutes before iC14 : 0. The GC spectra and the corresponding characteristic GC-MS spectra of iC14 : 1 and iC14 : 0 are shown in Fig. 4(b), (c) (marked by arrows). The mass difference of 2 indicated that the iC14 : 1

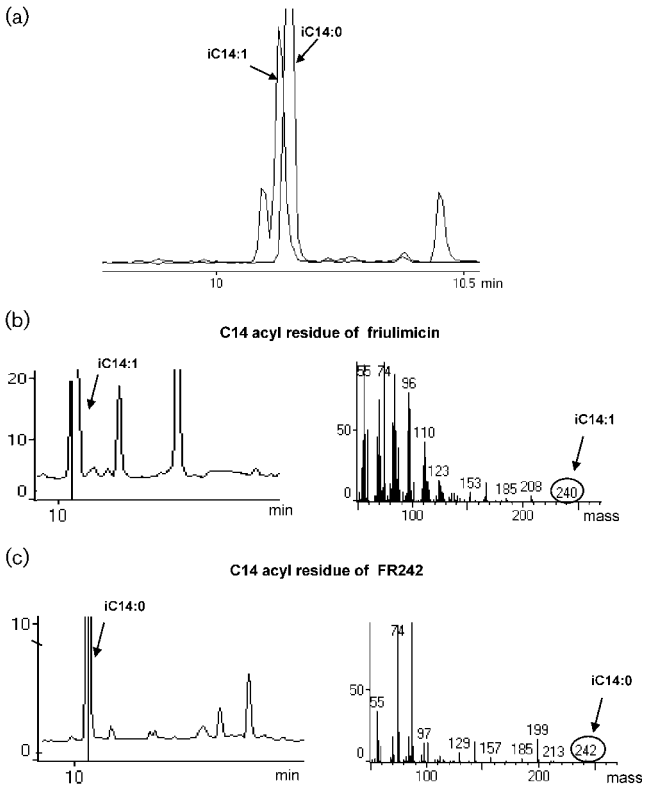


**Fig. 4.** GC analysis of the acyl residues of friulimicin derivatives. (a) Overlay of the iC14 : 0 and iC14 : 1 GC spectra of the acyl residues of FR242 and friulimicin, respectively, demonstrating the different elution times. (b) and (c) GC-MS analysis of the acyl residue of (b) friulimicin and (c) FR242. The GC spectrum of each acyl residue and the corresponding MS spectrum of the fatty acid of interest (marked by an arrow) with its mass number (circled) are shown.



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fatty acid of friulimicin possesses two hydrogen atoms fewer than the iC14 : 0 acyl residue of FR242, in other words, that the iC14 : 0 fatty acid is saturated. Therefore, the GC and GC-MS spectra showed that the acyl residue of FR242 from mutant MEHB lacks the characteristic Δ*cis*3 double bond, in other words, that LipB actually is responsible for or involved in the introduction of the double bond.

## Effect of hydrogenation of the acyl residue on biological activity

The double bond in the acyl residue of friulimicin apparently contributes to the activity of the antibiotic (see Fig. 3a). To analyse this in more detail, the fatty acid of native friulimicin was reduced with molecular hydrogen. Successful hydrogenation was verified by GC and GC-MS (data not shown). The same amount of hydrogenated friulimicin (corresponding to FR242) and native friulimicin were used in the bioassay. The zone of growth inhibition caused by hydrogenated friulimicin (FR242) was smaller than that caused by native friulimicin (approx. 63 % of the friulimicin inhibition zone; Fig. 3b), which is in agreement with the results of the bioassay using wild-type and mutant MEHB cells (Fig. 3a). Therefore, we postulate that the presence of the double bond enhances the action of the antibiotic.

## Heterologous expression of *lipB* in *S. lividans*

To demonstrate the assumed acyl-CoA dehydrogenase activity of LipB, we expressed the *lipB* gene heterologously in *S. lividans* T7, which carries the gene encoding T7 RNA polymerase under the control of the thiostrepton-inducible *tipA* promoter (Fischer, 1996). *S. lividans* T7 was transformed with the *lipB* expression plasmid pEHEX3 (Table 1). The expression of the *lipB* gene was under the control of the T7 promoter and resulted in the production of N-terminally His-tagged LipB protein (HisLipB) after induction with thiostrepton. HisLipB was purified by metal-chelate-affinity chromatography using Ni-NTA resin under native conditions, as described by Heinzelmann *et al.* (2001). The eluted fractions were analysed by SDS-PAGE (Fig. 5a). Using the spectroscopic acyl-CoA dehydrogenase assay according to the method of Kieweg *et al.* (1997) and Lea *et al.* (2000), no specific enzyme activity was detected when the protein-containing fractions were incubated with straight-chain activated fatty acids (nC14, nC15, nC16) – not even after modifying the reaction buffer conditions.

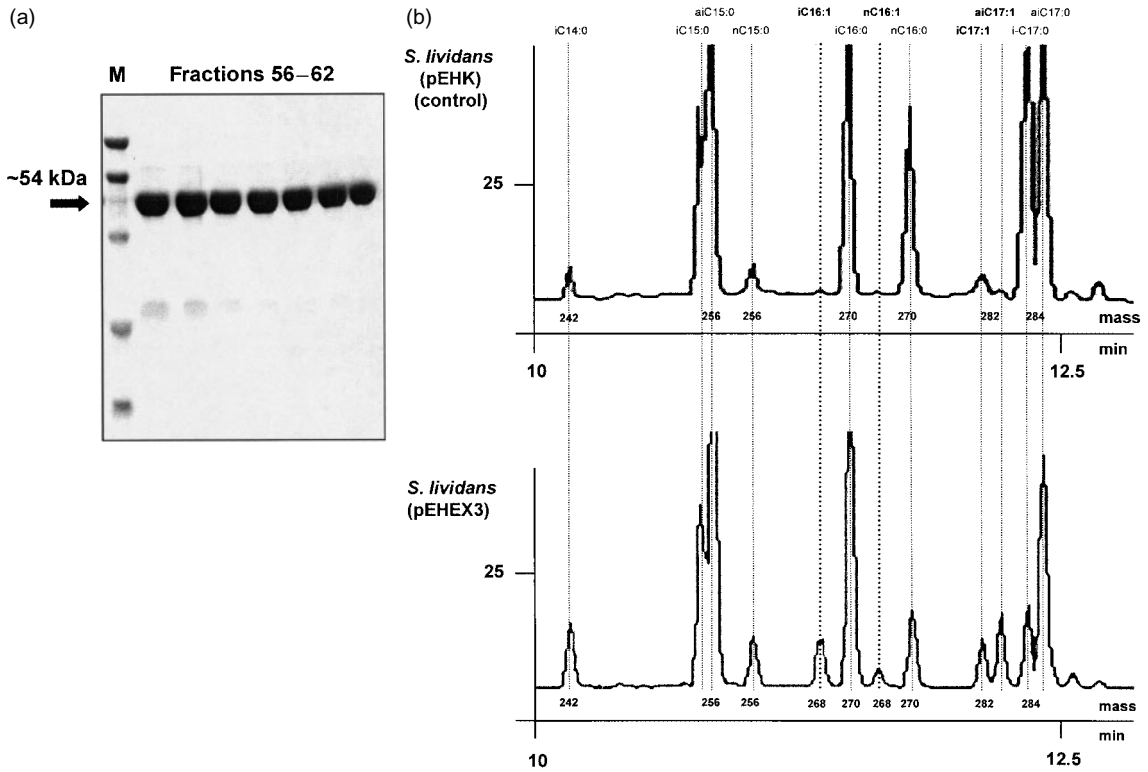


**Fig. 5.** Heterologous production of the His-tagged LipB protein in *Streptomyces lividans* T7. (a) Heterologous production of LipB was examined by SDS-PAGE after purification on Ni-NTA agarose. Lane M, SDS-PAGE molecular size standard, low-range (Bio-Rad); other lanes, the 54 kDa LipB protein eluted in fractions 56–62 during FPLC analysis. (b) Comparison of the GC spectra of *S. lividans* T7/pEHK and *S. lividans* T7/pEHEX3. Peaks of the *lipB* expression strain *S. lividans* T7/pEHEX3 not present or in increased concentrations compared to the control strain *S. lividans* T7/pEHK are indicated by the corresponding mass numbers from the MS spectrum.


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Therefore, it seems likely that under *in vitro* conditions, the substrate specificity of the putative acyl-CoA dehydrogenase is directed toward activated branched-chain fatty acids instead of activated straight-chain fatty acids. This is in keeping with the fact that branched fatty acids are attached to the peptide core of friulimicin. However, this hypothetical activity could not be biochemically investigated, since activated branched-chain fatty acids of a chain length of C13–C14 are not commercially available.

Therefore, we carried out an *in vivo* experiment using *S. lividans* T7/pEHEX3 and *S. lividans* T7/pEHK as a control (Heinzelmann *et al.*, 2003) with the aim of identifying differences in the fatty acid pattern of *S. lividans* T7/pEHEX3. Each of the two strains was cultivated in TSB medium, *lipB* expression was induced, and the fatty acid pattern was determined by GC-MS (Fig. 5b). In the GC spectrum of *S. lividans* T7/pEHEX3, two peaks not found in the spectrum of *S. lividans* T7/pEHK appeared: one in front of the iC16 : 0 fatty acid and the other in front of the straight-chain nC16 : 0 fatty acid (Fig. 5b). The corresponding mass spectra (data not shown) demonstrated that nC16 : 1/iC16 : 1 fatty acids with a mass number of 268 are formed, whereas the saturated nC16 : 0/iC16 : 0 fatty acids show a mass number of 270. In addition, the amount of iso- and anteiso-C17 : 1 (aiC17 : 1) fatty acids in *S. lividans* T7/pEHEX3 is higher than in the control strain, whereas the concentration of iC17 : 0 and aiC17 : 0 is lower (Fig. 5b). Therefore, we conclude that the LipB protein is involved in the formation of unsaturated fatty acids.

## DISCUSSION

The isolation of the DNA region flanking the previously isolated incomplete friulimicin biosynthetic gene cluster led to the identification of the novel biosynthetic gene *lipB*. The deduced LipB protein shows high similarity to acyl-CoA dehydrogenases, which are normally involved in an FAD-dependent oxidation step in the $\beta$-oxidation of fatty acids in which a double bond is introduced at position $\Delta$*trans*2 and FAD is reduced to $FADH_2$ (DuPlessis *et al.*, 1998; Hiltunen & Qin, 2000). In this paper, we show that LipB is involved in the introduction of the $\Delta$*cis*3 double bond into the acyl residue of friulimicin, as shown by analysis of the *lipB* mutant MEHB. A bioassay with friulimicin-sensitive *B. subtilis* showed that the zone of growth inhibition of mutant MEHB is approximately 38 % smaller than the zone caused by the wild-type (Fig. 3a). Since the zone of growth inhibition of chemically reduced friulimicin was approximately 37 % smaller than that of native friulimicin (Fig. 3b), it is likely that FR242, the friulimicin derivative of mutant MEHB, is less active than friulimicin. Furthermore, these results also revealed an effect of the $\Delta$*cis*3 double bond in the biological activity of friulimicin – the proposed hydrophobic interaction between the acyl residue and the C55-isoprenoid lipid carrier bactoprenylphosphate is possibly enhanced in the presence of the $\Delta$*cis*3 double bond. The $\Delta$*cis*3 fatty acid moiety of friulimicin might also affect the membrane fluidity in friulimicin-sensitive bacteria. Such an effect on membrane fluidity has been described in the context of the incorporation of shorter and $\Delta$*cis*-configured fatty acids into lipids in a species-specific manner as a response to cold-shock stress conditions (Weber *et al.*, 2001; Cropp *et al.*, 2000). Accordingly, the $\Delta$*cis*3 double bond in the acyl residue of friulimicin might enhance membrane fluidity and thereby the postulated interaction of the antibiotic with bactoprenylphosphate.

It is also possible that friulimicin [as described for daptomycin (Silverman *et al.*, 2003) or CDA (Lakey *et al.*, 1983)] is involved in $Ca^{2+}$-dependent pore formation, which leads to a disruption of the functional integrity of the cytoplasmic membrane. It can be speculated that the $\Delta$*cis*3 double bond of the acyl residue of friulimicin supports such a mechanism. Further experiments are needed to determine whether the haemolytic effect of FR242 on erythroblasts is also reduced.

The involvement of LipB in the introduction of double bonds in fatty acids was also confirmed by *lipB* overexpression in *S. lividans* T7. Under these conditions, higher amounts of unsaturated fatty acids were found in *S. lividans* T7/pEHEX3 than in the control *S. lividans* T7/pEHK (Fig. 5b). The major unsaturated fatty acids produced by *S. lividans*/pEHEX3 were iC17 : 1, aiC17 : 1 and iC16 : 1; nC16 : 1 was produced in lower amounts (Fig. 5b).

The *in vitro* spectroscopic enzyme activity assay with activated nC16 : 0 as substrate produced negative results, and the detection of nC16 : 1 was unexpected. The low concentration of nC16 : 1 produced *in vivo* suggests that oxidation of nC16 : 0 is only a side activity of the enzyme, which is probably not detectable in the *in vitro* enzyme assay using purified LipB.

Acyl-CoA dehydrogenases (ACADs) differ in their substrate specificity and are separated into short-chain (SCAD, $<$C6), medium-chain (MCAD, C6–C11), long-chain (LCAD, C12–C18) and very-long-chain (VLCAD, $>$C18) acyl-CoA dehydrogenases, which reflects the acyl-chain length of their preferred substrates (Lea *et al.*, 2000). According to this classification, LipB might be an LCAD that oxidizes fatty acids with a chain length of C12–C18. Structural analyses of different ACADs – SCADs, MCADs, and branched-short-chain acyl-CoA dehydrogenases, such as iso(3)valeryl-CoA dehydrogenase (i3VCD) – indicate that all members of this enzyme class show a characteristic polypeptide fold of 11 $\alpha$-helices (helices A–K) and 7 $\beta$-strands (1–7) (Kim & Miura, 2004). According to the determined protein structure, a substrate prediction seems to be possible for these enzymes (Kim & Miura, 2004).

The characteristic secondary structure of acyl-CoA dehydrogenases was also found in LipB [PROFsec (Rost & Sander, 1993; Rost, 1996), data not shown]. However, since no crystal structure data for straight long-chain and branched-long-chain acyl-CoA dehydrogenases are available, it would



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be helpful to solve the protein structure of LipB to determine the regions conferring substrate specificity.

Since only branched-chain fatty acids of a chain length of C13–C15 are attached to the peptide core of friulimicin (Vértesy *et al.*, 2000), a substrate specificity of LipB for branched-chain C13–C15 fatty acids was expected. However, why no iC14, iC15 and aiC15 : 1 fatty acids were found in *S. lividans* T7/pEHEX3 is unclear. It can be speculated that these fatty acids are formed and then degraded for unknown reasons. Such an instability and possible degradation of iC14, iC15 : 1 and aiC15 : 1 fatty acids has also been discussed by Cropp *et al.* (2000) regarding the induction of unsaturated fatty acid metabolism by cold shock in *S. avermitilis*. Another possible explanation for the lack of these fatty acids could be that the acyl-CoA synthase LipA, which probably activates fatty acids to form acyl-CoA thioesters, the substrates of acyl-CoA dehydrogenases (LipB), and which is essential for the synthesis of friulimicin (data not shown), has a narrow substrate range for branched-chain C13–C15 fatty acids. A possible LipA/LipB protein complex formation could prevent the oxidation and incorporation of other unsaturated fatty acids. A functional correlation of LipA and LipB is supported by the translational coupling of *lipA* and *lipB* in the friulimicin biosynthetic gene cluster. Similar situations have been observed in many antibiotic gene clusters, for example, within the biosynthetic gene cluster of the glycopeptide antibiotic balhimycin from *Amycolatopsis mediterranei*, in which the gene products of the four translationally coupled genes *dpgABCD* are involved in the synthesis of the unusual amino acid 3,5-dihydroxyphenylglycine (Pfeifer *et al.*, 2001).

The chemical structure of the acyl residue of FR242 in the *lipB* mutant indicates that the acyl-CoA dehydrogenase LipB is responsible for the introduction of the Δ*cis*3 double bond, but the mechanism is unknown. Two main pathways can be postulated for the formation of the Δ*cis*3 double bond in the acyl residue of friulimicin (Fig. 6): 1) LipB introduces the Δ*cis*3 double bond in one reaction by a new mechanism or LipB is a bifunctional enzyme with both dehydrogenase and isomerase activity (Fig. 6, pathway 1), or 2) LipB introduces the double bond in the Δ*trans*2 position as acyl-CoA dehydrogenases of primary metabolism and the double bond is then isomerized to the Δ*cis*3 position by an unknown Δ*trans*2-Δ*cis*3-isomerase, for which an encoding gene has not been identified within the biosynthetic gene cluster (Fig. 6, pathway 2). The activity of such an enzyme would be similar to that of FabM, a

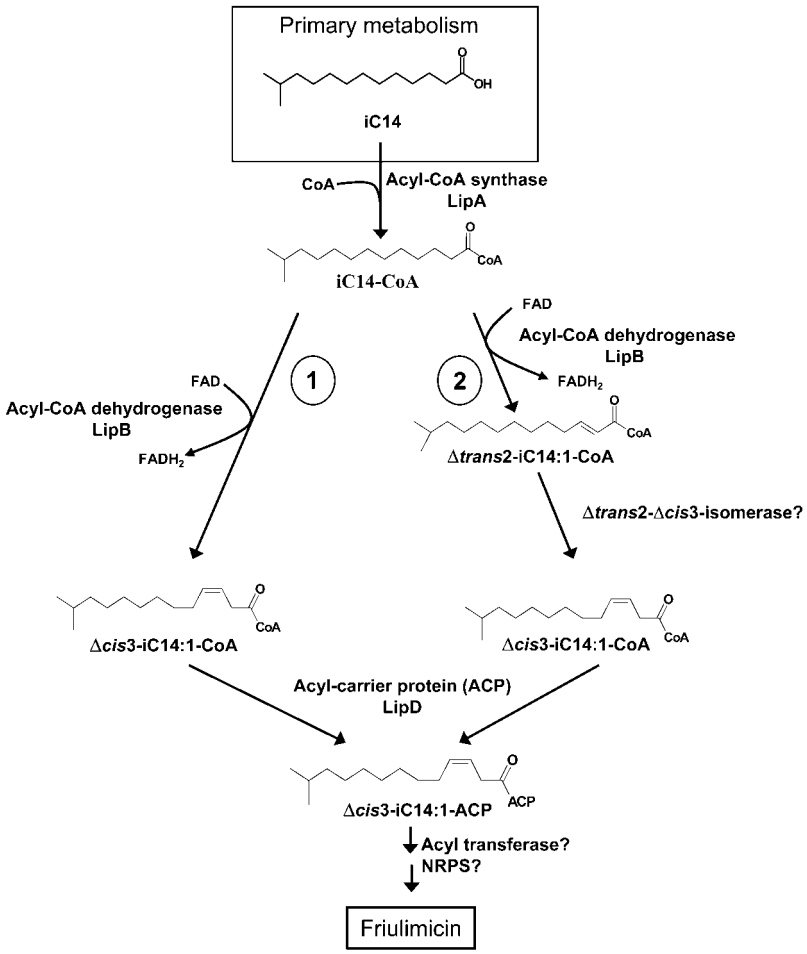


**Fig. 6.** Model of the acylation mechanism of the lipopeptide antibiotic friulimicin. NRPS, non-ribosomal peptide synthase.


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Δ*trans*2-Δ*cis*3-isomerase from *Streptococcus pneumoniae* (Marrakchi *et al.*, 2002).

Functions for the other Lip proteins in delivering the substrate of LipB and attaching the product of LipB to the peptide can be hypothesized. Since the acyl residue of friulimicin is similar to fatty acids of the fatty acid pool of *A. friuliensis*, which includes fatty acids of a chain length of C14–C18, the acyl residue might originate from primary metabolism. The putative acyl-CoA synthase LipA may be able to activate branched and long-chain fatty acids, iC14 to iC14-CoA (Fig. 6) for example, which are then converted to Δ*cis*3-iC14 : 1 by LipB. The 4′-phosphopantetheine cofactor of a putative ACP (LipD) could possibly then attach the fatty acid to the peptide core. However, it cannot be excluded that the acyl reside is linked to the first amino acid (asparagine or aspartic acid), which then is activated by a non-ribosomal peptide synthase (NRPS) and condensed with the other amino acids of the peptide.

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