A novel fusidic acid resistance gene from *Streptomyces lividans* 66 encodes a highly specific esterase

Beate von der Haar, Stefan Walter, Susanne Schwäpenheuer
and Hildgund Schrempf

Author for correspondence: Hildgund Schrempf. Tel: +49 541 969 2895. Fax: +49 541 969 2804.
e-mail: schrempf@sfbbio1.biologie.uni-osnabrueck.de

Universität Osnabrück, FB
Biologie/Chemie,
Barbarastraße 11, 49069
Osnabrück, Germany

Resistance to fusidic acid in *Streptomyces lividans* is due to secretion of an extracellular enzyme (FusH) that converts the steroid antibiotic into an inactive derivative. NH₂-terminal and several internal amino acid sequences were prepared from the purified enzyme. Using one of the deduced oligonucleotides to probe a subgenomic DNA library, the *fusH* gene was cloned and sequenced. Sequence analysis located an ORF which, owing to the presence of two putative start codons, indicates a predicted protein with 520 or 509 amino acids. A signal peptide was identified by aligning the deduced amino acids with the N-terminal sequence determined for the mature enzyme. The C-terminal part of the deduced FusH contains a region of three tandemly repeated stretches of 50 amino acids, which is preceded and followed by amino acids showing high homology with the repeats. FusH was found to share a GDS motif with some deduced esterases. *S. lividans* transformants carrying *fusH* on a multicopy vector synthesized high levels of FusH. Purified FusH cleaved equally well an acetyl, a thioacetyl or a formyl group from the 16 β -position of fusidic acid and its derivatives. However, a propionyl group at the 16 β -position was attacked with difficulty and a 16 α -acetyl group was not hydrolysed at all. These data indicate that FusH is a highly specific esterase. The *fusH* gene is widely distributed among streptomycetes that modify fusidic acid to its inactive lactone derivative.

Keywords: *Streptomyces lividans* 66, fusidic-acid-inactivating enzyme, esterase (FusH), *FusH* gene

INTRODUCTION

Fusidic acid is a steroid-like antibiotic isolated from the fungus *Fusidium coccineum* (Godtfredsen *et al.*, 1962). It is structurally related to helvolic acid from *Aspergillus fumigatus* (Waksman *et al.*, 1943) and cephalosporin P1 from *Cephalosporium* sp. (Burton & Abraham, 1951).

Fusidic acid binds to elongation factors EFG (Tanaka *et al.*, 1969) and EF2 (Malkin & Lipman, 1969) from prokaryotic and eukaryotic organisms, respectively. Spontaneous fusidic-acid-resistant strains from *Staphylococcus aureus* and certain *Escherichia coli* strains possess a modified EFG (Tanaka *et al.*, 1971; Chopra,

1976). Some *Staph. aureus* strains carry plasmids that encode fusidic acid resistance (Chopra, 1976).

Wild-type *Streptomyces lividans* 66 is resistant to fusidic acid. Comparison of this strain and a fusidic-acid-sensitive mutant allowed the identification of an extracellular enzyme that inactivates fusidic acid (von der Haar *et al.*, 1991). Using a combination of ultrafiltration and chromatography on Phenylsepharose and an anion exchanger, the enzyme was highly purified. Its apparent molecular mass is 48 kDa and its activity is optimal between 45 °C and 55 °C and between pH 6.0 and 9.0. It is stimulated by neither mono- nor divalent ions. The enzyme removes the acetyl group at C-16 from fusidic acid. The resulting intermediate is unstable and spontaneous lactonization between C-21 and C-16 occurs rapidly (von der Haar & Schrempf, 1995).

In this paper, we report the characterization and

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overexpression of the gene encoding the specific esterase that inactivates fusidic acid and some of its derivatives.

METHODS

Strains and plasmids. *Streptomyces lividans* 66 was kindly provided by D. A. Hopwood, Norwich, UK. *Streptomyces olivaceoviridis* (Schnellmann *et al.*, 1994) and *Streptomyces reticuli* and *Streptomyces scabies* have been described earlier (Schrempf, 1985). The *E. coli* plasmids pUC18 (Yanisch-Perron *et al.*, 1985) and pUS18 (Dittrich *et al.*, 1991) and the *Streptomyces*-*E. coli* shuttle vector pWHM3 (Vara *et al.*, 1989) were used. The construct pWF1 (see Fig. 2) consists of pWHM3 and a 3.2 kb *Bam*HI fragment on which the cloned *S. lividans* *fusH* gene is situated.

Culture conditions. Strains were grown on complete agar medium or in liquid minimal medium (von der Haar & Schrempf, 1995; Hopwood *et al.*, 1985). To attain optimal production of the inducible fusidic-acid-modifying enzyme, 3×10^7 spores *S. lividans*(pWHM3) or *S. lividans*(pWF1) ml⁻¹ were added to 2 l flasks containing 250 ml minimal medium and shaken for 24 h at 120 r.p.m. An additional 250 ml minimal medium and 50 µg fusidic acid (Sigma) were added to each flask and the mixture was subdivided into 250 ml volumes. Cultivation was then continued for another 36 h (von der Haar & Schrempf, 1995).

Purification of the enzyme. After the culture (500 ml) had been centrifuged at 10000 *g* for 20 min, the supernatant was concentrated by ammonium-sulfate precipitation (90%, w/v) and resuspended in 500 µl 20 mM Tris/HCl buffer, pH 7.0. Chromatographic purification of the enzyme was as recently described by von der Haar & Schrempf (1995).

Enzyme assays. For the determination of fusidic-acid-modifying activities, samples were diluted with 50 mM phosphate citrate buffer, pH 7, to a total volume of 100 µl and 100 µg fusidic acid was added. After incubation at 30 °C for 5 h, the antibiotic and the products were extracted twice with 0.5 ml ethyl acetate. The combined extracts were evaporated at 75 °C. The remaining substances were suspended in 10 µl ethanol and analysed by TLC, as described by von der Haar *et al.* (1991). The reaction products were quantified by scanning the chromatograms and subsequent densitometric evaluation of the spots with the computer program Cybertech CAM2.0. One unit of activity was defined as the conversion of 1 µmol fusidic acid to its inactive products per minute (von der Haar & Schrempf, 1995).

SDS-PAGE, transfer of proteins and amino acid sequencing. SDS-PAGE was performed with 10% polyacrylamide gels in the presence of 0.1% SDS according to the method of Laemmli (1970). If protein sequences were to be determined, the protein was blotted onto a PVDF membrane (Immobilon P, Millipore), as described earlier (Schlochtermeier *et al.*, 1992). The N-terminal sequences of the mature protein and of internal peptides generated by cleavage with LysC protease were determined by R. Schmid, Universität Osnabrück, Germany, and P. Jungblut, Wita, Germany, respectively.

Preparation of the oligonucleotide and hybridizations. The N-terminus of the longest internal peptide was used to synthesize a 39-mer oligonucleotide. The digoxigenin-labelled 39-mer oligonucleotide was hybridized at 64 °C with DNA fragments transferred from an agarose gel to a nylon membrane (Sambrook *et al.*, 1989). After 20 h at 64 °C, the nylon membrane was washed twice in 2× SSC (5 min at room

temperature) and twice in 0.1× SSC (15 min at 64 °C). Immunodetection was carried out according to the specifications of the DNA labelling and detection kit supplied by Boehringer.

Transformation of strains. *E. coli* was transformed with plasmid DNA by the CaCl₂ method (Sambrook *et al.*, 1989). *Streptomyces* protoplasts were prepared and transformed in the presence of polyethylene glycol according to the standard procedure (Hopwood *et al.*, 1985). An overlay of 0.4% agarose containing 500 µg thioestrepton ml⁻¹ was used to select transformants.

Isolation of DNA. Plasmids of *E. coli* and *Streptomyces* strains were isolated by the alkaline method with strain-specific modifications (Sambrook *et al.*, 1989; Hopwood *et al.*, 1985). Total DNA of *Streptomyces* strains was released by neutral lysis from cultures grown in a sucrose-containing complete medium for 2 d (Hopwood *et al.*, 1985).

Preparation and screening of a subgenomic DNA library. Total DNA (200 µg) from *S. lividans* was cleaved with *Sst*II and separated on preparative agarose gels. Fragments of about 1.3–1.5 kb were extracted from the gel and ligated to *Sst*II-linearized pUS18 (Dittrich & Schrempf, 1992). A subgenomic DNA library consisting of *Bam*HI fragments of 3–3.5 kb was obtained in a similar fashion, using *Bam*HI-linearized pUC18. Each of the ligation mixtures was used to transform *E. coli* XL1-Blue. Ampicillin-resistant transformants were tested for the presence of the desired insert by colony hybridization with the 39-mer oligonucleotide at 64 °C.

General DNA techniques. Modification of DNA with nucleases, polymerases and ligases was carried out by standard procedures (Sambrook *et al.*, 1989). DNA restriction fragments were resolved by agarose gel electrophoresis.

DNA was sequenced using the T7 sequencing kit from Pharmacia and digoxigenin-labelled oligonucleotides (MWG) that corresponded to primers of the *lacZ* system.

Computer analysis. Sequences were compared using the FASTA program and the SWISS-PROT and EMBL databases; they were analysed with the GENMON program (GBF), and reading frames were determined with the GCWIND program (D. Shields, Dublin, Ireland) on the basis of the codon usage preferences in *Streptomyces* DNA.

RESULTS AND DISCUSSION

Determination of amino acid sequences and identification of the associated gene

The enzyme FusH purified from *S. lividans* culture supernatant solutions was subjected to Edman degradation. The N-terminal sequence of the mature protein was determined to be XGEPAAATD. In addition, FusH was cleaved by the protease LysC and the N-termini of several internal peptides were analysed. The N-terminus (DLGRLYFADVDGDR) of the longest internal peptide obtained was used to deduce and synthesize the 39-mer oligonucleotide GAC CTS GGS CGS CTS TAC TTC GCS GAC GTS GAC GGS GAC. This was hybridized under various conditions with total DNA from *S. lividans* 66 cleaved with different restriction enzymes. As shown in Fig. 1, single *Bam*HI, *Sma*I, and *Sst*II fragments of the *S. lividans* genome hybridized with the 39-mer probe.

Fig. 1. Hybridization of the digoxigenin-labelled 39-mer oligonucleotide deduced from an internal peptide of purified FusH with *S. lividans* genomic DNA cleaved with *Sma*I (lane 1), *Bam*HI (lane 2) and *Sst*II (lane 3). Lane 4 contained *Hind*III-cleaved and digoxigenin-labelled λ DNA. The markers on the right indicate the calculated sizes of the *S. lividans* fragments.

Cloning and sequencing of the gene

A subgenomic DNA library in *E. coli* XL1-Blue containing 1.3–1.5 kb *Sst*II fragments of *S. lividans* 66 cloned in pUS18 was hybridized with the 39-mer oligonucleotide. Three transformants carried the expected constructs, each of them containing the same 1.4 kb *Sst*II insert. Because this insert was too small to contain the complete gene for FusH, the 3–3.5 kb fragments from *S. lividans* DNA cleaved with *Bam*HI were cloned in pUC18 and used to transform *E. coli* XL1-Blue. Colony hybridization was performed with the *Sst*II fragment as probe. Four transformants contained the expected construct carrying a 3.2 kb *Bam*HI insert in one or the other orientation. After colony hybridization and subsequent DNA analysis, the sequences of overlapping fragments obtained by subcloning were determined in both orientations (Fig. 2).

Analysis of the sequence and the deduced protein

One complete ORF was detected within the cloned sequence. It had a G + C content of 71.6 mol %, which is typical of most *Streptomyces* genes. Two possible start codons (GUG at position 206 and UUG at position 239) with corresponding Shine–Dalgarno sequences (Fig. 3a) and one stop codon (UGA) could be identified. Most *Streptomyces* genes contain the start codon AUG, but sometimes GUG and UUG are used (Thberge *et al.*, 1992; Fernndez-Abalos *et al.*, 1992). The sequenced ORF may encode a 55.3 or a 54.2 kDa protein with 520 or 509 amino acids, respectively. The pI of the mature protein was calculated to be 5.39. By aligning the deduced amino acid sequence with the amino acid sequence (IXGEPAAATD) determined by Edman degradation of the N-terminus of the purified mature protein, the cleavage site (AQA, consensus AXA) for a signal

Fig. 2. (a) Strategy for sequencing a 2.1 kb segment of the cloned 3.2 kb *Bam*HI fragment. (b) The hybrid plasmid pWF1, which consists of the shuttle vector pWHM3 and the 3.2 kb *Bam*HI fragment containing the *fusH* gene.

peptidase could be identified (Fig. 3a). The amino acid composition of the deduced signal peptide resembles that of other secreted enzymes. The predicted size of the mature protein is 50.7 kDa. Whether each of the start codons is used as a translational start is not known. A dual start motif has been identified in holin genes from lambdoid phages and in the *Bacillus* phage ϕ 29 (Tedin *et al.*, 1995), but is also known in streptomycetes (Raymer *et al.*, 1990).

Three directly repeated sequences are present within the gene (Fig. 3b). Aligning these regions in the deduced protein revealed three identical consecutive blocks of 50 amino acids. Moreover, this region is preceded by a stretch of 47 amino acids showing 47% identity, and followed by stretches of 40 amino acids showing 63% identity with the region repeated three times. Studies with the products of truncated forms of the gene will be necessary to determine the role of these repeats, which have not as yet been reported in other deduced proteins.

Our biochemical investigations (see below) suggested that FusH is a specific esterase. We therefore compared regions of FusH with sequences deduced from other

Fig. 3. (a) Nucleotide sequence of the *fusH* gene and the deduced amino acid sequence. The N-terminal amino acids of the internal peptides (determined by Edman degradation) are underlined. The N-terminus of the protein is indicated by a dotted line. The two putative start codons are shown in grey boxes and the two possible sequences corresponding to the 3'-terminal end of the 16S rRNA of *S. lividans* are double-underlined. (b) Alignment of repeated amino acid motifs within the FusH protein.

GEFVGNELIEFTRSVFPPIPKREKTFIFGGLSMGGYGAIRNGL	XynC
DEMAKYDLPASINFILNK-TGQEQVYVVGHSQGTTFGFIAFS	LipA
WTGNDYDTFADDTIAQLIEHLDLKEVTLVGFSMGGGDVARYIA	Pfa
DGKYSQARWVEHAIGVLDALGIQQGDIVGNSGGGLALALAI	XylF
DAQYNRDVWVDHAGVGLDALEIEQADLVGNSGGGIALALAI	DmpD
AVQQE-LLPLVKATAPFS-DRADRTVVAGQSGGLSALYAGL	Fes
RFRTVYAARDLAAALLDAA-GMPRPVVLGGQSMGNIQAQEYLR	NonR
FDQAAALEFVKSNITENFG-GDPNQITIWGYSAGAASVSQLTM	Est1
KDQVAALKWIIQQNIVAFG-GDPNSVTITGMSAGAESVHNHLI	EstE
KDQRLALKWIKQNIASFSG-GEFQNVLLVGHSAAGASVHLQML	Est6
KDQRLAIKWIIRNNIARFG-GDPHNIILLGFGSTGESSVHLQML	EstS
TDQVEALRWIADNVAAFG-GDPNRTILVGGSGGAYSTAALAQ	Pcd
RQDHMAIAWVKRNIAAFG-GDPDNITIFGSGAGASV-SLQT	Bal
FDQQLALQWVQKNIAAFG-GNPKSVTLFGESAGAGSV-GLHL	Chle
LDQIKALEWVYNNIGSFG-GNKEMITIWGESAGAFSV-SAHL	D2
LDQLAALRWVQDNIAAFG-GNPDSVTIFGESAGGVSV-SALV	Est1
LDQLAALRWVQDNIAAFG-GNPDSVTIFGESAGGVSV-BVLV	Est2
LDQAAALKWVRENISAFG-GDPDNVTVFGESAGGMSI-AALL	PnbA
LDQRLALQWVQENIAAFG-GDPTSVTLFGESAGAGSV-GMHL	Aces
AIDVIFDGLSSTTRSRVKPKDITIT--SGDSGGANLHLALS	Est
GDGRA-----DIVVIRENGDVVVRHNLGDSFLAAGRVMGGW	FusH
GDGRWMSLHHRFVADSKDKEPEVV--FLGDSLVLQMLHQCEIM	PAF
ALGSCLALAGPAGSAGAAPADPVPTVFFGDSYTANFGTAPVT	EstA
MIRLLSLVLFFCLSAASQASEKLLV--LGDSLSSAGYQMPIEK	VmA
GFGDSRTDKSNS	9-O-Ac-SA

Fig. 4. Alignment of amino acids of the GX₁SX₂G or GDS motif from various deduced esterases. XynC, *Caldocellum saccharolyticum* (Lüthi *et al.*, 1990); LipA, human (Anderson & Sando, 1991); Pfa, *Pseudomonas fluorescens* (Pelletier & Altenbuchner, 1995); XylF, *Pseudomonas putida* (Horn *et al.*, 1991); DmpD, *Pseudomonas putida* (Nordlund & Shingler, 1990); Fes, *E. coli* (Pettis *et al.*, 1988); NonR, *Streptomyces griseus* (Plater & Robinson, 1992); Est1, *Caenorhabditis elegans* (Kennedy *et al.*, 1993); EstE, *Myzus persicae* (Field *et al.*, 1993); Est6, *Drosophila melanogaster* (Collet *et al.*, 1990); EstS, *Drosophila virilis* (Sergeev *et al.*, 1993); Pcd, *Arthrobacter oxidans* (Pohlenz *et al.*, 1992); Bal, rat (Fontaine *et al.*, 1991); Chle, dog (Arpagaus *et al.*, 1991); D2, *Dictyostelium discoideum* (Bombliet *et al.*, 1990); Est1, rat (Takagi *et al.*, 1988); Est2, rabbit (Ozols, 1989); PnbA, *Bacillus subtilis* (Zock *et al.*, 1994); Aces, human (Soreq *et al.*, 1990); Est, *Acinetobacter calcoaceticus* (Reddy *et al.*, 1989); FusH, *Streptomyces lividans* (this paper); PAF, cow (Hattori *et al.*, 1994); EstA, *Streptomyces scabies* (Raymer *et al.*, 1990); VmA, *Vibrio mimicus* (Shaw *et al.*, 1994); 9-O-Ac-SA, influenza (Hayes & Varki, 1989).

esterase genes (Fig. 4). The active centres of several proteases, lipases, acetyltransferases and esterases known to be serine hydrolases consist of a triad of the amino acids serine, histidine, and aspartic or glutamic acid. In the crystal structure of an acetylcholine esterase (Sussman *et al.*, 1991) and lipases (Winkler *et al.*, 1990; Brady *et al.*, 1990), folding of the protein brings these residues close to each other. Within the primary structure deduced from esterase genes, however, only the serine residue of the active sites can be identified from its location in a GX₁SX₂G or a GDS motif. The FusH protein possesses the GDS motif (Fig. 4).

Expression of *fusH* in the *S. lividans* wild-type and a fusidic-acid-sensitive mutant

The 3.2 kb *Bam*HI fragment (Fig. 2) that contained the *fusH* gene was recloned in the multicopy *Streptomyces-E. coli* shuttle vector pWHM3 (Vara *et al.*, 1989), resulting in the multicopy construct pWF1. The constructs were used to transform the *S. lividans* wild-type strain and its spontaneous fusidic-acid-sensitive variant *S. lividans* 90-2. Thiostrepton-resistant transformants furnished the anticipated plasmids. Comparative studies revealed that wild-type *S. lividans* and *S. lividans*(pWF1) showed resistance levels of 15 and 70 µg ml⁻¹, respectively, to fusidic acid. In contrast, the *S. lividans* 90-2 mutant carrying pWF1 was resistant only to low levels (5 µg ml⁻¹) of fusidic acid. The data suggested that *fusH* was overexpressed when present on a multicopy vector in wild-type *S. lividans*, but not in the mutant *S. lividans* 90-2.

Proteins in culture filtrates of wild-type *S. lividans*, its

fusidic-acid-sensitive mutant *S. lividans* 90-2 and the corresponding transformants carrying pWF1 were concentrated and analysed. Fusidic-acid-inactivating activity was highest [1.3 mU (ml culture filtrate)⁻¹] in *S. lividans*(pWF1). The activity in strain 90-2(pWF1) was about 20-fold lower. The reaction products were extracted in ethyl acetate and analysed by TLC. Wild-type *S. lividans*, *S. lividans*(pWF1) and *S. lividans* 90-2(pWF1) converted fusidic acid (R_F 0.42) to two compounds of R_F 0.20 and R_F 0.61. The compound with R_F 0.20 had already been characterized as the enzymic reaction product that spontaneously converts to the lactone at R_F 0.61 (von der Haar *et al.*, 1995). Fractionation of the extracellular proteins by SDS-PAGE showed that *S. lividans*(pWF1) contained the largest quantity of a 48 kDa protein (Fig. 5). The size of this protein corresponded to that of FusH purified earlier (von der Haar *et al.*, 1995) from wild-type *S. lividans*, and to the size (50.7 kDa) calculated for the polypeptide deduced from *fusH* (see above). The data show that the cloned gene encodes a specific esterase which is overproduced if the gene is present in high copy number.

Transformants of the fusidic-acid-sensitive mutant *S. lividans* 90-2 showed only a very low level of esterase activity after transfer of precultured mycelia to media containing fusidic acid. The genome of *S. lividans* 90-2 contains the 3.2 kb *Bam*HI fragment on which the *fusH* gene is situated (hybridization data not shown). Therefore, unlike tetracycline- and chloramphenicol-sensitive variants in *S. lividans* (Kessler *et al.*, 1989; Dittrich *et al.*, 1992), the fusidic-acid-sensitive mutant does not arise by deletion of the complete *fusH* gene. Instead, it may be due to a mutation or deletion in a region required for

Fig. 5. (a) PAGE of concentrated protein extracts. Lanes: 1, *S. lividans*(pWF1); 2, *S. lividans*(pWMH3). The arrow indicates the band corresponding to purified FusH in lane 3. (b) TLC analysis of fusidic-acid-modifying activity. Lanes: 1, *S. lividans*(pWF1); 2, *S. lividans*(pWMH3); 3, fusidic acid (control). Strains were grown without fusidic acid for 24 h and then shifted to a medium containing 25 µg fusidic acid ml⁻¹ for 36 h. Proteins were precipitated with (NH₄)₂SO₄ and suspended in buffer containing fusidic acid. The reaction products were identified as described in Methods.

induction of the *fusH* gene. Further studies will be required to elucidate the regulation of *fusH*.

Substrate specificity of FusH

FusH cleaves the acetate group of fusidic and helvolic acids, but not of nitrophenyl, methylumbelliferyl or steroid esters (von der Haar & Schrempf, 1995). To elucidate the specificity in more detail, derivatives of fusidic acid (von Daehne *et al.*, 1979) were tested. The following results were obtained (Fig. 6). (i) An acetyl, a thioacetyl or a formyl group located in the 16β-position was cleaved off. (ii) A 16β-propionyl group was very slowly hydrolysed. (iii) Additional substituents in position 21 (i.e. glycine conjugated to fusidic acid) did not prevent hydrolysis of the 16β-acetyl group. (iv) In contrast to a β-acetyl group, an acetyl group in the α-position was not cleaved off. (v) Tetrahydrofusidic acid also served as a substrate.

Distribution of the *fusH* gene

Several streptomycetes modify fusidic acid in the same way as does *S. lividans* (von der Haar *et al.*, 1991). Evidence that *S. coelicolor* A3(2) DNA contains hybridizing fragments, the sizes of which are identical to those of *S. lividans*, suggests that these strains possess a very similar, if not identical, *fusH* gene. DNA fragments of varying sizes from several other strains modifying fusidic acid (*S. olivaceoviridis*, *S. scabies*, *S. reticuli*) hybridized with the *fusH* gene (data not shown). As fusidic acid is used to treat various infections caused by *Staph. aureus* and other micro-organisms, the possibility that these pathogens can acquire resistance due to the synthesis of a FusH-like activity deserves attention.

Fig. 6. (a) Structure of fusidic acid. (b) Fusidic acid derivatives and cleavage rates by FusH. A sample (50 µg) of fusidic acid or of its derivatives was incubated with the esterase for 5 h. After TLC analysis, the reaction products were quantified as described in Methods. The percentage of hydrolysis of various compounds was related to that of fusidic acid (100%). (c) Reaction mechanism of FusH. The structure of the lactone derivative was determined by von der Haar *et al.* (1991).

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