Biotransformation of fusidic acid and its related derivatives by *Streptomyces lividans*

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To my daughter Karla…..

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Abbreviations

A	absorbance
aa	amino acid(s)
acetyl-CoA	acetyl-Coenzyme A
ACD	acyl-CoA dehydrogenase
ACO	acyl-CoA oxidase
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CIP	calf intestinal alkaline phosphatase
CV	column volume
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EF-Tu	elongation factor Tu
EF-G	elongation factor G
FAD	flavin adenine dinucleotide
GTP	guanosine triphosphate
HIC	hydrophobic interaction chromatography
IEC	ion exchange chromatography
IEF	isoelectrofocusing
IPTG	isopropyl-ß-D-thiogalactoside
kb	kilo base(s)
kDa	kilo Dalton(s)
L-3-scHAD	hydroxyacyl-CoA dehydrogenase (L type)
mA	milliampere
Mb	Mega base(s)
MFE	multifunctional protein
mM	millimolar
MS	mass spectrometry
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
orf	open reading frame(s)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
Rf	reference value
rpm	revolutions per minute
RP-HPLC	Reversed phase high pressure liquid chromatography
RT	room temperature
TLC	thin layer chromatography
SDR	short-chain dehydrogenase/reductase
W	Watt
WT	wild type

I Introduction

1. Characteristics of streptomycetes

Members of the genus *Streptomyces* are Gram-positive bacteria, which belong to the family of *Actinomycetales* and are characterized by high G+C content (up to a 74%) (Stackebrandt and Woese 1981, Goodfellow and Cross 1984, Embley and Stackebrandt 1994). Their natural habitat soil is nutritionally, biologically and physically complex and variable, demanding for their fast adaptation. As a consequence, they are able to perform a broad range of metabolic processes, numerous biotransformations, as well as degradation of the world's most abundant biopolymers lignocelluloses and chitin (Ball et al. 1989, Wachinger et al. 1989). The development of streptomycetes comprises different life stages: substrate mycelia, aerial hyphae and chains of spores (Kelemen et al. 1998, Champness 2000). The timing of each stage and their switches are subtly controlled by a large variety of regulatory genes, induced or repressed upon extracellular signals. Studies of the two groups of *S. coelicolor* A3(2) developmental mutants: *bld* mutants, (failing to produce the aerial mycelia) and *whi* mutants, (aerial hyphae fail to complete the production of the normal grey spores) indicate a high complexity of regulatory network which includes morphogenesis and production of secondary metabolites (Chater 2001). The recently published complete genome sequence of the model strain *Streptomyces coelicolor* A3(2) revealed several more remarkable features and at the same time opened new questions (Bentley et al. 2002). The ~8.6 Mb linear chromosome belongs to one of the largest completely sequenced bacterial genome. Its replication is bidirectional, proceeding from the centrally located oriC to the chromosome termini (Zakrzewska-Czerwinska and Schrempf 1992). The terminal ends are replicated by so called "end patching", where DNA synthesis is primed by terminal proteins (Bao and Cohen 2001). The coding density is largely uniform across the chromosome, which contains remarkably 7825 predicted genes far more than the genome of *Escherichia coli* (4289 genes) or *Sacharomyces cerevisiae* (6203 genes). The genome also shows a strong emphasis on genes coding for putative regulators (12.3%) and 65 sigma factors. The ability to exploit a large variety of nutrients is supported with the 10.5% of potentially secreted proteins, since the majority of them are hydrolases (proteases/peptidases, chitinases, celullases and amylases). The distribution of known different types of genes discloses however a central core spanning about the half of the chromosome and a pair of arms. In this biphasic division nearly all important genes (like for cell division, DNA replication, amino acid biosynthesis etc.) are located in the stable central core, while the genes for the secondary metabolism and hydrolytic

enzymes lie in the arms. From 18 clusters that may be involved in the secondary metabolism, only three are related to antibiotic synthesis, whereas the others seem to be responsible for the biosynthesis of the "stress metabolites" like hopanoides, siderophores and others (Bentley et al. 2002).

The synthesis of secondary metabolites including antibiotic production in streptomycetes generally occurs in a growth phase-dependent manner (Demain and Fang 1995). For most of them the synthesis starts after the vegetative phase has ceased and streptomycetes start to develop aerial mycelia. Complementation of certain *bld* mutants (Lawlor et al. 1987, Elliot et al. 1998 and 2001, Bignell et al. 2000 and 2003) restores the antibiotic production, exerting their role as global regulators.

Antibiotic	**Target**	**Organism**	**Reference**
aminoglycosides: parmomycin, kanamycin, gentamycin hygromycin B	Binding to 30S ribosomal subunit and 16S RNA, causing misreading of the mRNA and blocking translocation	*S .kanamyceticus* *S. kanamyceticus* *S. hygroscopicus*	Moazed and Noller 1987 Jerinic and Joseph 2000 Schroeder et al. 2000
thiostrepton	Inhibit GTPase activity of the elongation factor EF-G	*S. azureus*	Thompson et al. 1993, Floss 1988, Cameron et al. 2002
tetracyclines	Binds to 30S ribosomal subunit and blocking binding the aa-tRNA at A site, also prevents binding of the release factors	*S. rimosus*	Ross et al. 1998, Brown et al. 1993, Brodersen et al. 2000
pactamycin	Initiation step by blocking the release of initiation factors from 30S initiation complex	*S. pactum*	Mankin 1997, Brodersen et al. 2000
kirromycin	Inhibiting elongation by stabilizing ternary complex EF-Tu:GDP: ribosome	*S. ramocissimus*	Vogeley et al. 2001, Olsthoorn-Tieleman et al. 2002
spectinomycin	Binds to 30S subunit and inhibits translocation of the peptydyl-tRNA from the A to the P site	*S. flavopersicus*	Bilgin et al. 1990, Lyutzkanova et al. 1997
puromycin	Causes premature termination of the nascent peptide by binding to A site	*S. alboniger*	Vara et al. 1988 Starck and Roberts 2002

Table 1 Examples of antibiotics of *Streptomyces* strains that inhibit protein synthesis

Concerning the structure, antibiotics exhibit a large variety of chemical classes like aromatic polyketides (oxytetracycline; *S. rimosus*, Butler et al. 1989, Findlow et al. 2003), macrolides (erythromycin; *S. erythrea*, Cortes et al. 1990, Carreras et al. 2002), a ß-lactam-type (cephalosporin *S. clavuligerus*, Demain and Piret 1991), aminonucleoside (puromycin; *S. alboniger*, Vara et al. 1988, Starck and Roberts 2002), aminoglycosides (streptomycin; *S. griesus* Ohnuki et al. 1985, Gharaibeh et al. 2003), peptide antibiotics (thiostrepton; *S. azureus*, Cameron et al. 2002), and chromopeptides (actinomycin; *S. antibioticus*, Jones and Hopwood 1984). Many of them target specific sites on the translational complex, causing the inhibition of protein synthesis at different stages (Table 1).

Antibiotic–encoding genes are mostly clustered with their regulatory genes and one or more resistance genes, the later ones are responsible for the protection against the own antibiotic. As an example, the oxytetracycline (*otc)* biosynthetic genes are clustered and flanked by two resistance genes (*otr*A and *otr*B) in a 34 kb segment of the *S. rimosus* chromosome (Butler et al. 1989, McDowall et al. 1999). The *otr*A, which shows high similarity to *tet*M (*Streptococcus* sp.) codes for the ribosomal protection protein whose NH_2-terminal part is highly homologous to the elongation factors (Doyle et al. 1991, Schnappinger and Hillen 1996, Chopra and Roberts 2001). The second resistance determinant, *otr*B codes for a deduced protein which is a close homologue to the *E. coli* membrane associated TetB efflux pump. This was shown to function as electroneutral antiporter, exporting the antibiotic complexed with a metal ion (like Mg^{2+}) in proton exchange across the cell membrane (Schnappinger and Hillen 1996, Chopra and Roberts 2001). This type of resistance seems to be common to streptomycetes, and includes secretion of other tetracyclines (*S. ambofaciens*), landomycin (*S. cyanogens*) granaticin (*S. violaceoruber*) and puromycin (*S. alboniger*) (Lee et al. 2003). The similarity among polyketide and the fatty acid-biosynthesis supports the idea that genes for the secondary metabolites have evolved from those for the primary ones. The differences emanate in the type of the used starter units (polyketides use malonate, acetate, propionate or butyrate), the condensation length, stereochemistry and the choice of the tailoring enzymes (Katz and Donadio 1993, Donadio and Sosio 2003). The evolved enzymes often have intrinsic substrate tolerance as shown by experiments using “nonnatural” substrates for polyketide synthases and their associated modifying enzymes (hydroxylases, oxidases and glycosyltransferases) resulting in the differently modified polyketides (Pohl 2002). Glycosyltransferases attach to the cyclic skeleton a large variety of 6-deoxysugars leading to newly glycosylated forms of avermectin (Wohlert et al. 2001); landomycin (Trefzer et al. 2001) and tetracenomycin (Rodriguez et al. 2002). In addition to antibiotics, streptomycetes

produce a large range of pigments, cytostatics, antifungal substances etc. Some secondary metabolites function as chemical signals or hormones (γ-butyrolactones), being able to induce species-specific differentiation (Beppu 1992, Chater and Horinouchi 2003). So far there are few published examples of using streptomycetes in targeted biotransformation of metabolites. One example is the use of *S. griseolus* for biotransformation of prosulphuran (pesticide) to give products arising from the hydroxylations of the C3 and C4 triazine methyl group and the benzylic carbon of the trifluoropropyl substituent (Holland 1998). The bioconversion of the antitumor substance taxol/cephalomannine by *Streptomyces* sp MA7065 results in hydroxylation of the 10-acetyl-methyl group at the side chain of the benzene ring (Chen et al. 2001). Reversible oxidation of various steroid hormones and their derivatives by different hydroxysteroid dehydrogenases from *S. hydrogenans* has been also reported and includes the action of 20ß-hydroxysteroid oxidoreductase (Szymanski and Furfine 1977), 3α,20ß-hydroxysteroid dehydrogenase (Strickler et al. 1980), 17ß-hydroxysteroid dehydrogenase (Märkert and Träger 1975) and 3α-hydroxysteroid dehydrogenase (Ghosh et al. 1992)**.** They all catalyze the reversible oxidation of related hydroxyl groups of the steroid hormones in the presence of the NAD(H). As their homologous mammalian counterparts they belong to the short-chain dehydrogenase/reductase superfamily. The high sequence divergence is reflected by only five conserved residues in all members of the family, including the "YxxxK" catalytic sequence (Tyr152 and Lys156) (Duax and Ghosh 1997). The importance of the steroid biotransformations refers to the fact that the steroid hormones which are involved in a wide range of physiological processes (like blood pressure, cancerogenesis, and alcohol metabolism) are (in)activated by mammalian steroid dehydrogenases (Duax et al. 2000).

2. Fusidic acid, mode of the action and evolved resistance

Fusidic acid (FA) is a steroid-like antibiotic, originally isolated from the fermentation broth of the fungus *Fusidium coccineum* (Godtfredsen et al. 1962), has frequently been used during the last 40 years against penicillin-resistant staphylococcal infections. It has also been proven to be efficient against numerous pathogenic bacteria including *Nocardia* sp. (Black and McNellis 1970), *Staphylococci* sp. (Fleurette et al. 1989), *Mycobacterium* sp. (Witzig and Franzblau 1993), *Clostridia* sp. and *Actinomyces* sp. (von Daehne et al. 1979). Its therapeutical usefulness is also based on the excellent distribution in various tissues, low toxicity, lack of allergic reactions and absence of the cross resistance with the other commonly used antibiotics (Christiansen 1999, Turnidge 1999). Structural analyses showed that it is identical to the described antibiotic ramycin isolated from *Mucor ramannianus*

(Vanderhaeghe et al. 1965, Jannsen and Vanderhaeghe 1967). The other members of steroid-like antibiotics are helvolic acid produced by *Aspergillus fumigatus* or *Cephalosporium caerulens* (Okuda et al. 1964), cephalosporin P1 produced by *Cephalosporium acremonium* and viridominic acid A, B and C synthesized by *Cladosporium sp*. (von Daehne et al. 1979) (Fig. 1.1).

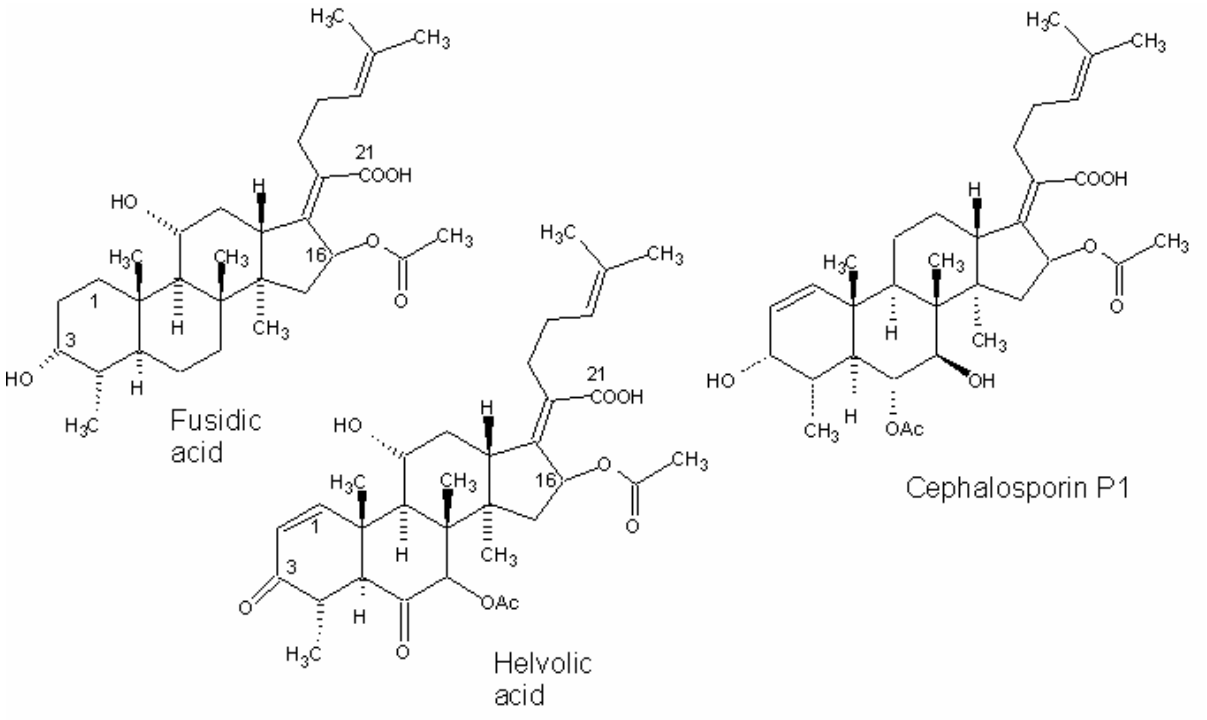


Figure 1.1 Chemical structures of steroid-like antibiotics fusidic acid, helvolic acid and cephalosporin P1

The unique feature of this class of antibiotic is the tetracyclic skeleton, confining unusual chair-boat-chair (*trans-syn-trans*) conformation, which is different from the common steroids (*trans-anti-trans*) (Godtfredsen et al. 1965a, b). These compounds also have in common the unsaturated side chain with the carboxylic group linked to the steroid ring at C17 via a double bond and the acetate group at C16. The extensive structure-activity relationship studies on the derivatives of fusidic acid, its closely related helvolic acid and cephalosporin P1, defined those parts of the molecule which are essential for varying degree of antibacterial activity (von Daehne et al. 1979, Godtfedsen et al. 1966). The least important part of the molecule is the lipophilic side chain, whose modifications mostly led to the active derivatives, but the Δ^{17} double bond proved to be essential for the activity by providing the correct geometry of the side chain in respect to the carboxylic group (Duvold et al. 2001). The most essential parts are the carboxylic group at C20, the ß-acetate group at C16 with its correct configuration (ß

versus α) and the tetracyclic fusidane skeleton. Those substituted hydroxyl groups which are functionally related in helvolic acid and cephalosporin P1 can be replaced by other functional groups (keto, halogens, sulfoxides and azides) retaining the same or slightly reduced activity when compared with the FA (von Daehne et al. 1979, Duvold et al. 2001).

FA inhibits the protein synthesis of pro- and eukaryotes at the translocation step during elongation of the nascent polypeptide, by binding to the elongation factor EF-G (or EF-2) and stabilizing ternary complex EF-G-GDP-ribosom. EF-G participates in the elongation phase of protein synthesis by translocating the peptidyl-tRNA from the A site to the P site (Kaziro 1978). It is supposed that the EF-G-GTP complex binds to a defined site on the ribosome in the pre-translocation state. After GTP hydrolysis to GDP and P_i and resulting translocation the EF-G:GDP loses its affinity for the ribosome (Stark et al. 2000, Rodnina et al. 1997). As a consequence of the FA binding, the ribosome is blocked in the ternary complex and cannot achieve the posttranslocational conformation inevitable for the next cycle of elongation. At the same time EF-G occupies the space on the ribosome which overlaps with the binding site of the elongation factor EF-Tu (Berchtold et al. 1993). The exact mode of FA action and its binding site on EF-G remains unclear (Lauberg et al. 2000, Martemyanov et al. 2001, Agrawal et al. 1998, Stark et al. 2000, Czworkowski et al. 1994, Evarson et al. 1994). The elongation factor EF-G from *Thermus thermophilus* is a large GTPase (661 amino acids), folded in five domains, and is structurally similar to the binary complex of the EF-Tu-tRNA (Czworkowski et al. 1994, Nissen et al. 1995, Berchtold et al. 1993). One EF-G mutant lacking domain 4 is impaired in translocation, does not have contact with the α sarcin loop of the 23S RNA and is not able to leave the ribosome upon the GTP hydrolysis, indicating that the contact with the α sarcin loop or its conformational change is required for the EF-G-GDP release (Rodnina et al. 1997). Analysis of the numerous FA resistant mutants of *Staphylococcus aureus* revealed single amino acid substitutions in the EF-G (Lauberg et al. 2000), located mostly in domain 3 and few in domain 1 and 5. Mostly they are on the interfaces of these domains. FA binds stoichiometrically to the ribosomal complex (Okura et al. 1971). Moreover EF-G lacking domain 3 lost GTPase activity but is able to bind to the ribosome in the presence of the GDP or GTP and FA (Martemyanov et al. 2000). These findings indicate that many mutations affect EF-G conformation but not the direct binding of the FA molecule. One possible binding site for FA could be located between domains 5 and 6 near the GTP binding center (Czworkowski et al. 1994), thereby preventing interdomain motion (G/domain2 versus domain3/domain5). Other proposed FA binding sites are also in

the vicinity of the GTP binding center (Evarson et al. 1994) or in the area of the effector loop (residue 40-65) (Lauberg et al. 2000) (Fig 1.2).

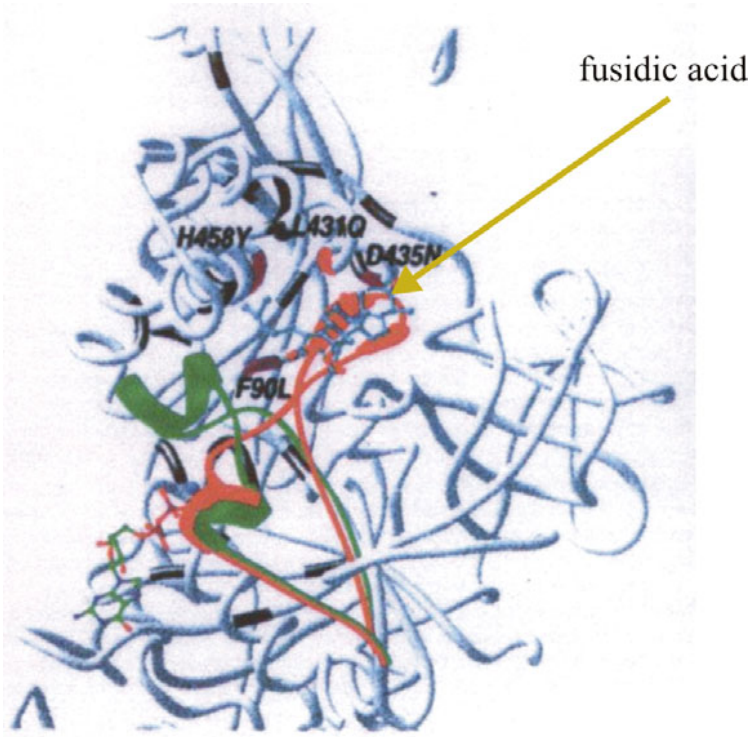


Figure 1.2 Putative binding site of the fusidic acid

EF-G point mutations (conferring the strong resistance towards the fusidic acid) in the violet (*S. aureus*) or red (*S. typhimurium*) are clustered in the helix C_G and helix B_3. The effector loop of EF-Tu was superimposed according to the identity to the EF-G effector loop, since the later one remained unresolved. The EF-Tu in GDP conformation (red) and EF-Tu:GDPNP:Cys-tRNA^{cys} (green). Putative position of the fusidic acid is shown by arrow (Lauberg et al. 2000).

Binding of FA within the proposed sites could lead to conformational changes affecting GTP-binding and EF-G movement (Lauberg et al. 2000, Czworkowski et al. 1994, Johanson et al. 1996). Therefore it was proposed that FA binds to the EF-G after GTP hydrolysis (Willie et al. 1975).

Chloramphenicol resistance in *E. coli* strains is based on enzymatic modification by chloramphenicol-acetyltransferase which is also responsible for one type of the FA resistance. The later mechanism is not based on enzymatic modification, but rather on tight binding of the FA to the chloramphenicol-acetyltransferase (Marcoli et al. 1980, Völker et al. 1982, Bennet and Shaw 1983).

Apart from the common fusidic acid resistance based on the modification of the elongation factor as a target site, there is an enzymatic inactivation of the FA, performed by the secreted esterase FusH from *S. lividans* (von Haar et al. 1991, 1995 and 1997). The enzyme FusH deacetylates the FA at C16 giving rise to inactive and unstable 16ß-OH deacetyl fusidic acid, which spontaneously converts into its inactive lactone derivative (Fig. 1.3).

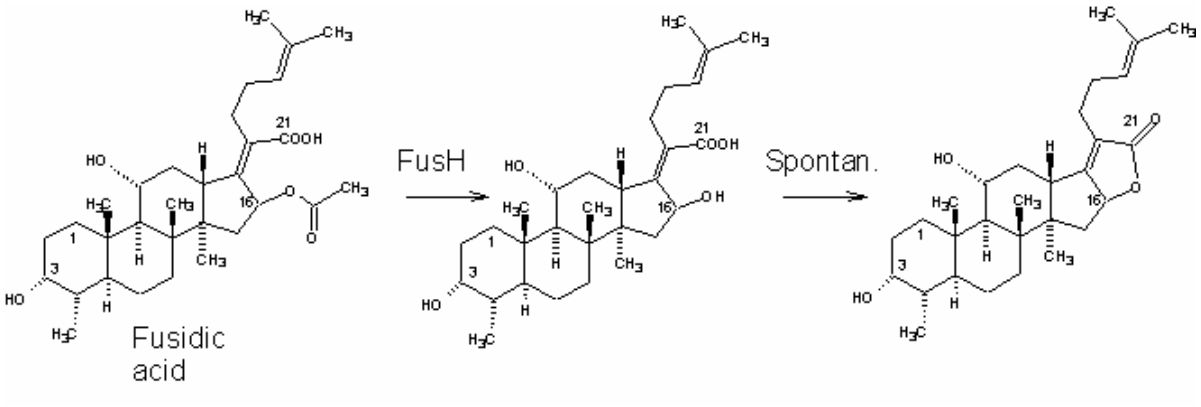


Fig 1.3 Enzymatic inactivation of the fusidic acid by esterase FusH (von Haar et al. 1997)
The second step leading to the lactone is supposed to take place spontaneously.

Sequence analysis of the 48 kDa FusH showed that it contains a characteristic "GDS motif" common to other esterases (von Haar et al. 1997). The enzyme exhibits substrate and stereospecificity, hydrolyzing only 16ß-acetyl, formyl and thioacetyl derivatives, but very slowly propionyl derivatives. The 16α-acetyl derivative was not hydrolyzed at all. This kind of protection seems to be widespread at least among the streptomycetes (von Haar et al. 1997). Suspicions that some pathogens may acquire the same kind of defense was confirmed by Harada and coworkers (1999), who found that *Nocardia sp* in the presence of fusidic acid (FA) produces two inactive products: the lactone already described in *S. lividans* (von Haar et al. 1997) and its 7α-hydroxylated derivative.

Sequencing the DNA neighbouring the *fus*H gene disclosed the presence of several genes in its vicinity, which may be involved in fusidic acid modification. One of them, the *fus*T may code for a transmembrane protein, while the *fus*A gene is found to code for an enzyme related to the acyl-CoA dehydrogenases (Richter, Diploma Thesis 1999).

The aim of my studies was to explore, whether *S. lividans* has the capacity for additional reactions leading to biotransformation of fusidic acid and to characterize the enzyme(s) that may be involved.

II Materials and Methods

1. Chemicals and Enzymes

ACL, UK	Affinity dye ligand chromatography PIKSI kit
	Matrices: blue1 and orange3
Biomol,USA	Pefablock SC
BioLab New England, USA	Quick Ligation kit
	Molecular weight protein marker-8S (6.5-175 kDa)
Cleveland Ohio, USA	Polymerase
Detroit, Michigan, USA	Casamino acids
	Yeast extract
	Trypton
Difco Laboratories, USA	Agar
Fluka-Riedel-de Haën, Germany	Glutaraldehyde (50% v/v)
	1,4-dithio-D,L-threitol
	$K(H_2PO_4)$ (HPLC)
	$K_2(HPO_4)$ (HPLC)
	Coomassie Brilliant Blue R-250
Gibco-BRL Ltd, Eggenstein	Restriction enzymes
	T4-Ligase
	Taq-Polymerase
Henselwerk GmbH, Germany	Soja meal
J.T. Baker Holland	Acetonitril (HPLC)
	Methanol (HPLC)
Macherey-Nagel GmbH, Düren	Nucleobond AX Kit PC 100
	RP-HPLC C18 (5µm, 250x4 mm)
Merck, Darmstadt, Germany	Aluminium and glass TLC plates: Kieselgel 60 F_{254}
	Dimethylsulphoxide
Novagen, (Merck), Germany	pET Cloning kit
PALL GmbH, Germany	Fluorotrans membrane (PVDF)
Amersham, Biosciences, Germany	Ion exchange matrices: Diethylaminoethyl (DEAE)
	Carboxymethylcelullose (CMC), MonoQ
	Hydrophobic matrices: Phenyl-sepharose (Fast flow), Phenyl-resource, Gel filtration Superdex 75

Promega, USA	*Pfu*I Amplification kit
Qiagen, Hilden, Germany	Ni-NTA agarose
	Ni-NTA-Alkaline Phosphatase conjugate
	Plasmid isolation kit, DNA fragment isolation kit
	Ni-NTA-Magnetic agarose beads kit
Roche, Mannheim, Germany	ATP
	Alkaline Phosphatase (Shrimp)
	DNAse, RNAse A
	Klenow-Enzymes
	Lysozyme
	NADH, Na salt
	NADPH, Na salt
	Restriction enzymes
	Sybr green I
Roth GmbH, Germany	Ammonium sulphate
	bis-Acrylamide
	bis-Tris
	Chloroform
	Comassie Brilliant Blue R250
	Ethylacetate
	Isopropyl-ß-D-thiogalactopyranosid (IPTG)
	n-Dodecylsulfate Sodium(SDS)
	Triton X-100
	Tween 80
Serva, Germany	Agarose (DNA grade)
	Acrylamide
	Ammoniumpersulphate
	Ampicilin trihydrate
	Anode buffer pH 3 and Cathode buffer pH 10
	Coomassie G250 and Blue W
	Chloramphenicol
	Dialyzing tubes
	Kanamycin sulphate
	Naphtol-AS-E-Phosphate

	Piperazine
	Servalyt precoates pH 3-10 and 3-5 (150 µm, 125x125 mm)
Sigma Chemical, Australia	Bromphenolblue
	FAD, Na salt
	Fusidic acid
	Isovaleryl-CoA
	Molecular weight protein marker-7B (27-180 kDa)
	NAD, Na salt
	Oligonucleotides for PCR
	Phenazine methosulphate
	TES
Sqipp, E.R. & Sons, Inc. USA	Thiostrepton
USB, USA	Bovine Serum Albumin (protease free)

The restriction endonucleases and the corresponding buffers were bought from the following companies: Roche Diagnostics GmbH (Mannheim), Life Technologies Gibco BRL (Eggenstein) MBI Fermentas GmbH (St. Leon Rot) and New England BioLabs GmbH (Frankfurt am Main). All other chemicals which were not mentioned here are products of the company Merck, Darmstadt, Germany and Sigma-Aldrich, USA.

2. Bacteria, Plasmids and Oligonucleotides

2.1 Bacteria

Bacterium	Geno-/Phenotype	Reference
E. coli DH5α	F^-, ϕ80d/*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-,m_K^+), *sup*E44, *rel*A1, *deo*R, Δ (*lac*ZYA-*arg*F) U169	Hanahan 1983
E. coli BL21	F^-, *omp*T, *hsd*S_B(r^-, m^-), *gal*, *dcm* (DE3) pLysS (Cm^R)	Grodberg and Dunn 1988
S. lividans 1326	wild type	Lomovskaya et al. 1972

2.2 Plasmids

2.2.1 Plasmid vectors

Plasmid	Size	Selection	Reference
pDrive	3.85 kb	*bla, kan,* ori-f1, ori-pUC	Qiagen
pWHM3	7.1 kb	*tsr, bla, ß-gal,* ori-pUC, ori-pIJ101	Vara *et al.* 1988
pET21a	5.44 kb	*bla,* T7-prom, *lac*I, ori-f1, 6xhistidine codons, ori-pBR322	Novagen
pET24a	5.31 kb	*kan*R, T7-prom, *lac*I, ori-f1, 6xhistidine codons, ori-pBR322	Novagen

2.2.2 Recombinant plasmids

Plasmid	Size	Genotype	Description	Reference
pBSK5	8.4 kb	*bla,* ori-pUC, *fus*A	5.5 kb *Bam*HI containing the *fus*A gene	Richter,Diploma Thesis 1999
pUKS10	7.23 kb	*bla,* ori-pUC, *fur*S, *cpe*B	4.6 kb *Kpn*I/*Sph*I containing *fur*S and *cpe*B genes	Zou et al. 1999
pDFA	7.74 kb	*ΔfurS- fus*A*, *bla*	PCR cloning of 1.72 kb *Sgf*I/*Xho*I *fus*A* in pUKS10	This work
pWFA1	12.2 kb	*ΔfurS-fus*A*	*Eco*RI/*Hind*III (Δ*fur*S-*fus*A*) from the pDFA cloned in pWHM3	This work
pTFA1	7.04 kb	*fus*A*	PCR cloning of 1.67 kb *fus*A (*Nde*I/*Xho*I) in pET21a	This work
pDFG1	4.72 kb	*fus*G	PCR cloning of the 873 bp *fus*G in pDrive	This work
pTFG2	6.16 kb	*fus*G*	PCR cloning of 913 bp *fus*G (*Nde*I/*Xho*I) in pET24a	This work
pWFG1	8.21 kb	*fus*G*	PCR cloning of 1.045 kb *fus*G* *Bam*HI/*Xba*I in pWHM3	This work

*- additionally codons for 6 histidines at 3' end of the corresponding gene

2.3 Oligonucleotides

Designation	Sequence	Purpose
FA*Sgf*I	5'-GTCGCGATCGCTGATGACGGTAGGTTCACGTC-3'	Cloning the *fus*A in pDFA
FA*Xho*I/His	5'-CACCTCGAGTCAGTGGTGGTGGTGGTGGTGGAC CACGGGCCGCG-3'	Cloning the *fus*A in pDFA
PUDAF	5'-CGGGACGGCGACCACCTCGG-3'	Forward pr. for sequencing the insert in pDFA
PUDAR	5'-GCCCTTGCCGGTGCCGTAGGTG-3'	Reverse pr. for sequencing the insert in pDFA
pDa int forw1	5'-CGGCGGCGTTCCTGATGTGG-3'	Internal primer for sequencing the insert in the pDFA
pDa int rev1	5'-CCCTCCCAGACCGAGTTCAG-3'	Internal primer for sequencing the insert in the pDFA
FGpWHM3f	5'-CCACGGTCGCCAGCCACATCC-3'	Forward primer for cloning in the pWHM3
FGpWHM3r	5'-GCTTCTAGATCAGTGGTGGTGGTGGTGGTGGCCG TAGGCGTAG-3'	Reverse primer for cloning in the pWHM3
FG*Nde*I	5'-ACCCTCATATGCCTGCGACCAGCCG-3'	Forward primer cloning the *fus*G in pET24a
FG*Xho*I	5'-CTGCTCGAGTCAGTGGTGGTGGTGGTGGTGGCC GTAGGCGTAGAA-3'	Reverse primer for cloning the *fus*G in pET24a
FG1	5'-GACCGGCATCTCGGGAGATAT-3'	Forward primer for cloning the gene *fusG* in pDrive
FG2	5'-CAGCCGTAGGCGTAGAAGCCAG-3'	Reverse primer for cloning the gene the *fusG* in pDrive
M13/pUC$_{forw}$	5'-GTAAAACGACGGCCAGT-3'	Forward primer for sequencing *fus*G in pDrive
M13/pUC$_{rev}$	5'-GTTTTCCCAGTCACGAC-3'	Reverse primer for sequencing *fus*G in pDrive

3. Media and Buffers

3.1 R-S Medium (Hopwood et al. 1985)

-For cultivation of streptomycetes.

K_2SO_4	0.25 g
$MgCl_2$ x 6 H_2O	10.12 g
Glucose	10 g
Casamino acids	0.1 g
Yeast extract	5 g
Tris	3.03 g
Serva-Agar (for solid media)	14 g

Bidest H_2O was added to 1.0 l and adjusted to pH 7.6. After autoclaving, the solutions below were added.

Trace element	2 ml
KH_2PO_4 (0,5 %)	1 ml
$CaCl_2$ x 2 H_2O (5 M)	0.4 ml

3.2 R+S Medium

-For the regeneration of *Streptomyces lividans* protoplasts.

R-S Medium was added with 103 g/l sucrose, Difco-Agar and 5.73 g TES (5.73%) or Tris. After autoclaving, the medium was supplemented with:

Trace element solution (1x)	2 ml
KH_2PO_4 (0,5 %)	10 ml
$CaCl_2$ x 2 H_2O (5 M)	4 ml
L-Proline 20 %	15 ml

3.3 Complete Medium (Jasenka Pigac)

-Liquid medium for cultivation of streptomycetes.

Sucrose	100 g
Tryptic Soy Broth	20 g
Yeast Extract	5 g

Filled with bidest H_2O to 990 ml. After autoclaving, supplemented with:

$MgCl_2$ x 6 H_2O (5 M)	10 ml

3.4 Soja-Mannit Medium

-For keeping streptomycetes on plates and for their sporulation.

Soja	20 g
Mannit	20 g
Agar	15 g

Filled with bidest H_2O to 1 l and adjusted to pH 7.6.

3.5 Minimal Medium

-For cultivation of streptomycetes.

KH_2PO_4	1.5 g
K_2HPO_4	2.0 g
$(NH_4)_2SO_4$	1.4 g

Add 1 l bidest H_2O pH 7.2.

After autoclaving, supplemented with:

$MgSO_4$ (10%)	1 ml
$CaCl_2$ (10%)	1 ml
Trace element solution (1x)	2 ml

Depending on the purpose, different carbon sources were added.

3.5 Trace element solution (10x)

-Used for R-S, R+S, Minimal medium.

$ZnCl_2$	40 mg
$FeCl_3$ x 6 H_2O	200 mg
$CuSO_4$ x 2 H_2O	10 mg
$MnCl_2$ x 4 H_2O	10 mg
$Na_2B_4O_7$ x 10 H_2O	10 mg
$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	10 mg

Filled with bidest H_2O to 100 ml, sterilized with filtration.

3.6 LB Medium (Luria-Bertani) (Sambrook et al. 1989)

-For keeping or cultivating *E.coli*.

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar (for solid medium)	14 g

Add 1 l bidest H_2O, pH 7.5

3.7 Frequently used buffers

Buffer	Content and the pH
TE	10 mM Tris-HCl pH 8; 1 mM EDTA pH 8
TBE	0.89 M Tris-HCl, 20 mM EDTA, 0.89 M borate pH 8
5xloading buffer	0.5xTBE, 0.25% Bromphenolblue, 50% glycerine
CAPS buffer	10 mM CAPS (3-(cyclohexylamino)-1-propanesulphonic acid, 10% methanol, pH 11
10xPBS	1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4
4xProtein Loading buffer	0.25 M Tris-HCl, 8% SDS, 20% 2-mercaptoethanol, 40% glycerol, 0.1% Bromphenolblue, pH 6.8
Staining buffer PAGE)	0.2% Coomassie R250, 50% methanol, 10% acetic acid

4.0 Culture conditions

4.1 *Streptomyces lividans*

4.1.1 Cultivation for the isolation of FusG and FusB

The minimal medium supplemented with the 0.5% yeast extract was used to cultivate *S. lividans* 1326 for the native isolation of FusG and FusB. A large amount of spores (10^{6-7} spore/ml) were inoculated in the 200 ml medium (5 µl/ml fusidic acid) and firstly left as a standing culture for 18-22 h /30^{o}C, and then shaking continued for 24 h (~130 rpm/min). The mycelia were harvested by centrifugation, washed with excessive amount of the minimal media and shifted in the 400 ml minimal media supplemented with the 1% glycerol and 30 µg/ml fusidic acid. Cultivation proceeded for 30-36 h, shaking (110 rpm/min), before continuing with the protein isolation.

4.1.2 Cultivation for the isolation of FusA-His tag, FusG-His tag and FusK

The complete medium was used to cultivate *S. lividans* transformants containing either cloned *fus*A-6xhistidine codons (*fus*A*) or *fus*G-6xhistidine codons (*fus*G*), [isolation of FusA-His tag (FusA*), FusG-His tag (FusG*)] and for isolation of FusK. A large amount of spores (10^{6-7} spore/ml) were inoculated in the 100 ml medium and 5 µl/ml fusidic acid and kept standing for 18-22 h at 30°C. Additional fresh 100 ml medium was added and antibiotic concentration was adjusted to final 5 µg/ml fusidic acid and/or 5 µg/ml thiostrepton (for the pWFA transformants to isolate FusA* and FusK or pWFG transformants for isolation of FusG*), and then left another 24 h shaking (rotary shaker 125 rpm/min) for mycelia propagation. After growth, the young mycelia were harvested by centrifugation and washed with excessive amount of minimal media, and then transferred in 400 ml minimal media supplemented with 0.5% yeast extract, 30 µg/ml fusidic acid and 5 µg/ml thiostrepton; cultivation by shaking (110 rpm/min) was proceeded for the next 24 h, before continuing isolation of the expressed proteins.

4.2 *E. coli*

E. coli strains DH5α or BL21 were cultivated in LB- medium at 37°C for the isolation of the plasmid DNA (Sambrook et al. 1989). Cultivation of the *E. coli* BL21 for the heterologous expression of FusA-His tag (FusA containing 6 histidines at C-terminus, named FusA*) and FusG-His tag (FusG containing 6 histidines at C-terminus, named FusG*) was modified in comparison to the standard procedure described in "pET System Manual" from the Novagen (www.novagen.com). According to the tested conditions temperature of cultivation, time of induction, IPTG concentration (see results), the optimal procedure was the following: the 10 ml LB medium was inoculated with the single colony and left growing overnight (17 h/ 37°C-starter culture). The 500 ml LB media containing the appropriate antibiotics (34 µg/ml chloramphenicol and 100 µg/ml ampicillin (pTFA1) or 30 µg/ml kanamycin (pTFG1) were inoculated with the 3 ml of the starter culture and shaking (120 rpm/min at 37°C) continued until the OD_{600} is 0.5. IPTG (final concentration of 1 mM) was added and the cultivation continued at 30°C- 120 rpm/min, for another 3 hours. Then the proteins were isolated (see 5.2.1).

4.3 Selection

For the cultivation of the *E. coli* strain containing one of the plasmids pDrive, pET21a, pWHM3 or constructs derived from them, 100 µg/ml ampicillin was added to LB liquid and

solid medium, for strains containing plasmid pET24a and its constructs 30 µg/ml kanamycin was added to LB liquid and solid medium. For the expression host *E. coli* BL21 34 µg/ml chloramphenicol was used in either liquid or solid medium. For *S. lividans* having pWHM3 based constructs, 5 µg/ml of a final concentration of thiostrepton was added to liquid medium, and 25 µg/ml in solid medium.

5. Purification of proteins

5.1 Purification of FusG and FusB

After propagation, the culture filtrate was separated from mycelia by centrifugation (20 min., 10^4xg), and further consecutive filtration through paper filter (Schleicher and Schuell), and cellulose acetate filters (1.2 µm and 0.4 µm, Schleicher and Schuell). To the clear supernatant $(NH_4)_2SO_4$ (final concentration of 0.7 M) was added and the pH 6.5 was adjusted with the bis-Tris, filtered once more through the cellulose acetate filter (0.2 µm Schleicher and Schuell) and loaded onto HIC-Phenyl sepharose Fast flow (Pharmacia HR 16/20) previously equilibrated with 0.7 M $(NH_4)_2SO_4$ in 20 mM bis-Tris pH 6.5. Proteins were eluted with a descending linear $(NH_4)_2SO_4$ gradient (0.7 M-0) with 6 column volumes (CV) at a flow rate of 2 ml/min. Fractions containing FusB activity were pooled, dialyzed (Serva dialyzing tubes, 50x volume, 4 h/ 4°C) against 25 mM Na-phosphate buffer pH 6 (Affinity blue1 chromatography) or 20 mM piperazine-HCl pH 5.5 (Affinity orange3 chromatography). After filtration via cellulose acetate (0.2 µm Schleicher and Schuell) the sample was applied with the flow rate 0.3 ml/min on dye ligand affinity chromatography columns (ACL HR 5/5) either blue1 or orange3 previously equilibrated with the starting buffers. (A) blue1: After adsorption, the column was excessively washed with 100 mM NaCl in 25 mM Na-phosphate buffer pH 6, then the unspecifically bound proteins were eluted from the matrix with washing (10 CV) 50 mM Na-phosphate buffer pH 8. The remaining proteins including FusB were eluted with 5 ml 5 mM FAD in 50 mM Na_2PO_4 pH 8. (B) orange3: The unspecifically bound proteins were washed away by replacing the 20 mM piperazine-HCl pH 5.5 buffer with the 60 mM Na-phosphate buffer pH 8 at a flow rate of 0.3 ml/min. The column was once more washed with the 1 ml (1 CV) of 1 M NaCl and the specifically bound proteins were eluted with the 5 ml 5 mM FAD, 1 M NaCl in 60 mM Na-phosphate buffer pH 8.

FusG was purified to apparent homogeneity in two steps: (**1**) HIC chromatography step is the same as for the partial purification of FusB (see above). The same protein pool, showing FusB activity, (HIC Phenyl sepharose Fast flow, see above) was dialyzed against 25 mM piperazine-HCl pH 5.5 (Serva dialyzing tubes, 50x volume, 4 h/ 4°C) and after filtration it

was applied at the flow rate 0.5 ml/min to (**2**) affinity chromatography column orange3, previously equilibrated with 25 mM piperazine-HCl pH 5.5. The weakly bound proteins were released by replacing the buffer with 60 mM Na-phosphate buffer pH 8. Since FusG binds more tightly to the orange3 than the other proteins, the column was additionally washed with 5 CV 1 M NaCl. The remaining pure FusG was eluted from the matrix by 5 ml 5 mM FAD, 300 mM NaCl in 60 mM Na-phosphate buffer pH 8.

5.2 Purification of FusA-His tag and FusG-His tag

5.2.1 Purification from *E. coli*

Proteins were purified under the native conditions. The *E. coli* strain carrying either pTFA1 (FusA*), or pTFG2 (FusG*) was propagated and induced as described in the section 4.2. The 3 g wet cells obtained by centrifugation (20 min, 10^4xg) were resuspended into 15 ml of the lysis buffer (300 mM NaCl 50 mM Na-phosphate buffer pH 8) containing 1 mg/ml lysozyme and 0.2 mM protease inhibitor (Pefablock SC) and left on ice 30 minutes. The cells were further sonicated (Branson Sonifier B12, 75 W): duty cycle 30%, output 2, time 10x10 seconds/ 0^{o}C, pause 10 seconds and the lysis was monitored by light-microscopy. The soluble proteins were separated from the remaining cell debris by centrifugation (30 min, 30000xg) and after adding imidazole (15 mM final concentration), the 20 ml protein solution was mixed with 1.5 ml (50% w/v) Ni-NTA matrix previously equilibrated in lysis buffer (binding phase) and then left shaking for 1 h/ 4^{o}C. Unspecifically bound proteins were removed from the Ni-NTA matrix by excessively washing (10 volumes) with the lysis buffer containing 25 mM imidazole and 300 mM NaCl. The bound FusA* or FusG* was stepwise eluted from the Ni-NTA matrix using 100 mM and 250 mM imidazole in 50 mM Na-phosphate buffer pH 8 containing 300 mM NaCl.

5.2.2 Purification from *S. lividans*

5.2.2.1 Purification of FusA-His tag

The enzyme FusA-His tag (FusA*) which is localized intracellularly can be purified to homogeneity in two steps: (**1**) DEAE chromatography. The mycelia gained from the liquid culture after the centrifugation (20 min, 10^4xg) were washed once with excessive amount of the 50 mM Na-phosphate buffer pH 8 and left shaking in (10 vol/mycelia weight) 300 mM NaCl, 0.1% Triton-X100 in 50 mM Na-phosphate buffer pH 8 for 30 min/ 4^{o}C, in order to release mycelia associated proteins. After centrifugation, 3 g of mycelia were washed two times with the 50 mM Na-phosphate buffer pH 8, and resuspended in 15 ml 50 mM Na-phosphate buffer pH 8 containing 0.2 mM Pefablock. After adding 2 mg/ml lysozyme and

incubation for 30 min at 4°C, the mycelia were subjected to the sonication (duty cycle 40%, output 4, time 10x15 seconds/ 0°C, pause 10 seconds), and the lysis was monitored by light-microscopy. After centrifugation (30 min 30.000xg/ 4°C) the cleared supernatant containing soluble proteins was directly loaded onto the DEAE (Pharmacia HR 10/10) column previously equilibrated with the 50 mM Na-phosphate buffer pH 8 at the flow rate of 1.5 ml/min. The bound proteins were eluted with 10 CV using a linear gradient 0 to 500 mM NaCl. Protein fractions containing FusA*, and NaCl between 100 and 300 mM were pooled, and after adjusting imidazole to 25 mM and NaCl to 500 mM, the pool was subjected to the batch Ni-NTA affinity chromatography. A 20 ml protein probe was mixed with 1.5 ml (50% w/v) Ni-NTA (previously equilibrated with 50 mM Na-phosphate buffer pH 8) for 1 h/ 4°C. Then the Ni-NTA matrix was filled into the column, washed with 10 CV of 50 mM Na-phosphate buffer pH 8 containing 30 mM imidazole and 500 mM NaCl. FusA* was eluted stepwise with 5 CV of 50 mM Na-phosphate buffer pH 8 containing 250 mM imidazole and 500 mM NaCl.

5.2.2.2 Purification of FusG-His tag

Since FusG-His tag (FusG*) was localized extracellularly and intracellularly, the culture filtrate and the mycelia served as starting material for the protein isolation. In both cases FusG* was purified in one step using optimized conditions for Ni-NTA affinity chromatography. After centrifugation (20 min, 10^4xg) and filtration, the proteins from the culture filtrate were precipitated with 90% w/v $(NH_4)_2SO_4$ and redissolved in 50 mM Na-phosphate buffer pH 8. In order to get rid off the traces of $(NH_4)_2SO_4$, the protein sample was additionally dialyzed (50x volume) against the same buffer 2 h/ 4°C. Before mixing the sample with the equilibrated Ni-NTA matrix [1.5 ml (50% w/v) per 20 ml of the protein probe] for 1 h/ 4°C, imidazole (25 mM final concentration) and the NaCl (500 mM final concentration) were added. After binding, the matrix was washed stepwise with 500 mM NaCl in 50 mM Na-phosphate buffer pH 8 containing 25, 50 or 75 mM of imidazole (5 CV). The purification of the intracellular FusG* was identical to the Ni-NTA purification of FusA* from the *S. lividans*, except that the bound FusG* was stepwise eluted (5 CV) with 500 mM NaCl in 50 mM Na-phosphate buffer pH 8, containing subsequently 25, 50 and 75 mM imidazole.

5.3 Purification of FusK

To the unbound protein fraction (flowthrough) obtained from the Ni-NTA purification step of FusA* from the *S. lividans* (see 5.2.2.1**),** $(NH_4)_2SO_4$ was added to a final concentration of 1.2

M. After centrifugation (20 min, 10^4xg) the protein solution was loaded at a flow rate 1.5 ml/min onto a column HIC Phenyl sepharose Fast flow (Pharmacia HR 10/10) previously equilibrated in 1.2 M $(NH_4)_2SO_4$/ 25 mM Tris-HCl pH 7.5. Proteins were eluted with the same buffer in a descending salt gradient (0.72 M-0 M) at a same flow rate in 10 CV. The active fractions which were eluted in the range of 0.25 M-0.12 M $(NH_4)_2SO_4$ were pooled, and after adjusting the salt concentration to 1 M $(NH_4)_2SO_4$ they were loaded onto HIC Phenyl resource (Pharmacia HR 5/5) column (equilibrated in 1 M $(NH_4)_2SO_4$/ 25 mM Tris-HCl pH 7.5). The proteins were separated by a linear descending salt gradient (0.6 M-0 M) at a flow rate 1 ml/min in 15 CV. The active fractions which were eluted with 0.3 M $(NH_4)_2SO_4$ were collected, 10x diluted with the starting buffer (25 mM Tris-HCl pH 8) and directly applied onto the equilibrated MonoQ (Pharmacia HR 5/5) column at a flow rate 1.5 ml/min. The proteins were released by an ascending linear gradient of NaCl (200 mM- 450 mM) in 25 mM Tris-HCl, pH 8, in 18 CV at a flow rate 1 ml/min.

5.4 Gel filtration

Gel filtration of the protein probe (500 µl) was done using column Superdex 75 (Pharmacia) in 150 mM NaCl- 25 mM Na phosphate buffer pH 6 at flow rate 0.8 ml/min.

6. Biochemical methods

6.1 Determination of the protein concentration

The protein concentrations of the samples were determined by the methods established by Lowry et al. (1951), or Bradford (1976).

6.2 SDS-Polyacrylamide and DNA gel electrophoresis

Preparation of protein probes and their separation using SDS-PAGE [11% (w/v), of polyacrylamide gel, pH 7.5 (resolving part) and 4% (w/v) polyacrylamide gel pH 6.8 (stacking part)] were done according to the Laemmli (1970). Used standard protein markers were 8S (kDa 175; 83; 62; 47.5; 32.5; 25; 16.5 and 6.5) and 7B (kDa 180; 116; 84; 58; 48.5; 36.5 and 26.6). DNA fragments are separated in a horizontal electrophoresis chamber (for example Pharmacia G-200) and visualized using Sybr Green I according to the method described by the company Roche (http://biochem.roche.com).

6.3 Electroblotting

The proteins were resolved by SDS-PAGE as described previously (Laemmli 1970). The gel was assembled together with prewetted Whatman paper and Fluorotrans membrane in freshly made CAPS solution (10 mM CAPS, 10% methanol pH 11) according to the manufacturer's protocol (PALL, Germany) using a semidry electroblotter (CTI, Idstein Germany). Blotting conditions were 1 mA/cm^2 for 1.5 h. The NH_2-terminal amino acid sequence was determined by Edman degradation, by Dr. H. Hippe, company Chromatec, Greifswald.

6.4 Protein staining after SDS-PAGE

Protein gels were stained either by the Coomassie staining procedure (Sambrook et al. 1989) or for higher sensitivity by silver staining procedure (Serva, especially noted in results). IEF gels were stained with the Blue W (Serva).

6.5 Western blot

In order to specifically detect proteins with antibodies, proteins were transferred onto the Fluorotrans membrane (PVDF, Pall, Germany) as described in 6.3. Then it was soaked in the 1xPBS containing 5% skim milk for 1 h/RT to saturate free binding sites. Further, the membrane was incubated in 1xPBS containing 1/1000 diluted primary *anti*-FusH antibodies, (von Haar et al. 1997) for 2 h at room temperature (RT), washed twice with 1xPBS for 15 min/RT and transferred into 1xPBS containing the secondary antibodies *anti*-rabitt IgG conjugated to alkaline phosphatase diluted to 1/5000, for 4 h/RT. After washing the membrane twice with the 1xPBS, it was shortly incubated in 25 mM Tris-HCl pH 8.3, and then changed to buffer (25 mM Tris-HCl, pH 8.3, 0.2 mg/ml naphthol-AS-E phosphate and 1 mg/ml Fast Violet) and left until the colour was developed. The reaction was terminated by washing the membrane in an excessive amount of water. Detection of the His-tag part of the transferred proteins was done using the alkaline phosphatase coupled with the Ni-NTA, according to the manufacturer's protocol (Qiagen).

6.6 Isoelectrofocusing

Native isoelectrofocusing (IEF) was done using the Servalyt precoates (150 µm, 125x125 mm, Serva) pH 3-5 or 3-10 in horizontal IEF chamber (Pharmacia) according to the protocol of the manufacturer for the IEF gels (Serva). The running conditions were the following: 200 V-3 mA-6 W for 15 min/ 4^{o}C and 2000 V-3 mA-6 W for 2 h and 15 min/ 4^{0}C.

6.7 Enzyme assays

Conversions of fusidic acid and its intermediates: lactone, substances A and B, 16ß-OH derivative were done according to the optimized conditions for every individual enzyme indicated in the corresponding chapter under Results. The assays were extracted by ethylacetate and analysed as described in 7.1 (Materials and methods). Isovaleryl–CoA dehydrogenase assay was done according to the protocol described by Zhang et al. (1999). Separation of the substances by RP-HPLC was modified according to the used column. The reaction mixture (100 µl) contained 100 mM phosphate buffer pH 8, 1.33 mM PMS, 0.4 mM FAD, 1 mM isovaleryl-CoA and the protein probe and incubated at 37^{o}C/ overnight. An aliquot of the enzyme assay was mixed 1:1 with the running buffer and applied onto the HyPurity Elite C18 RP-HPLC (3 µm, 150x4 mm column, ThermoQuest, USA). Substances from the assay were separated by isocratic elution (50 mM K-phosphate buffer, pH 5.7: methanol = 50:50).

7. Chemical methods

7.1 TLC analysis of modified products

Reaction products gained by catalysis mediated by FusG(His-tag), FusA-His tag, FusK or FusB were extracted by ethylacetate and separated by thin layer chromatography (TLC), Kieselgel 60 (Merck) by using chloroform:methanol = 9:1. Separated substances were visualized by spraying with 20% H_2SO_4 in methanol and subsequent heating at 110°C for 10 min.

7.2 Purification of the fusidic acid intermediates

For structural analyses the lactone derivative and the substances A and B were purified from the culture filtrate of *S. lividans* by ethylacetate-extraction. The organic phase was treated with the dry Na_2SO_4 (to get rid off the traces of the water) and after filtration it was concentrated by rotational evaporation. The crude concentrate was dissolved in a small volume of running solvent (dichloromethane: methanol = 97:3) and applied on an equilibrated Flash chromatography column (silica Kieselgel 60 H, 60x60 mm, pressure 1 bar). The substances were separated by stepwise elution dichloromethane:methanol = 97:3, then 90:10 and 70:30. The rest of the substances (higly adsorbed) were released by the ratio 50:50.

7.3 Reversed phase HPLC

All three substances were preparatively purified on the RP-HPLC (ThermoQuest, USA) using a C18-LiChrosorb column, (5 µm, 250x4 mm, Macherey-Nagel, Düren) and an isocratic elution acetonitril: water = 80:20 for the lactone isolation, or 65:35 for the isolation of the substances A and B. For analytical purposes (testing the enzyme FusG), substances A and B (which were obtained by enzymatic conversion of the lactone with FusB) were extracted by ethylacetate and then purified on the RP-HPLC using a HyPurity Advance column (3 µm, 150x4 mm, ThermoQuest) with isocratic elution methanol: water = 65:35.

7.4 Chemical analyses by mass spectrometry and NMR ^{1}H and ^{13}C

Pure substances lactone derivative, substances A and B were subjected to the mass spectrometry and NMR (^{1}H and ^{13}C). Data analysis and the structural elucidation have been done by Dr. H. Kasch and Dr. B. Liedke, Hans Knöll Institute, Jena.

8. Genetic methods

8.1 Isolation of chromosomal DNA from *S. lividans* and plasmid DNA from *E. coli*

The mycelia of *S. lividans* were obtained after the growth in a sucrose containing complete medium (Jasenka medium) for 2 days, first as a standing culture for 20 h/ 30^{0}C and then additionally shaking for another 24 h at 30^{0}C. The mycelia were separated by centrifugation (20 min, 10^{4}xg) and the following chromosomal DNA isolation was done according to the protocol from Hopwood et al. (1985).

The plasmids from *E. coli* were isolated using the Qiagen Plasmid Isolation Kit and corresponding protocols (Qiagen, www.qiagen.com).

8.2 Cleavage of DNA, purification of the fragments and ligation

The DNAs were cleaved with various restriction enzymes according to the suppliers' instructions. Gel electrophoresis was performed in 0.8-1% agarose gels using TBE buffer. DNA fragments were visualized under UV after staining with Sybr Green I (Roche) and purified by Qiaex Purification Kit (Qiagen). The ligation was performed with Quick T4 ligase (New England Biolabs) according to the manufacturer's protocol.

8.3 Transformations

E. coli was transformed with plasmid DNA using the $CaCl_2$ method (Sambrook et al. 1989), or by electroporation (Dower et al. 1988) using a Gene Pulser (Biorad). *S. lividans* protoplasts

were transformed and regenerated as described (Hopwood et al. 1985). *S. lividans* transformants were selected using an overlay of 0.4% agarose containing 500 µg/ml thiostrepton (Hopwood et al. 1985).

8.4 Polymerase Chain Reaction (PCR)

The amplification of the DNA fragments by PCR was done using Trio-Thermocycler (Biometra Göttingen). The time and temperature of the individual PCR steps (initial denaturation, alignment, polymerization, and cycle denaturation) including the cycle number were optimized for each individual case according to the characteristics of the template DNA and the used primers as indicated in the chapter of results The primers were designed using the program Vector NTI 6 and purchased from the company Sigma Ark, Steinheim (www.sigma-ark.com).

8.5 DNA sequencing and computer analysis

For sequencing specific DNA fragments generated by PCR were subcloned into pET21a/24a, pDrive and pUKS10. Non-radioactive PCR sequencing was performed using standard primers or primers based on the vector sequences (see Oligonucleotides) with the following parameters: Initial denaturation 96^{o}C/3 min, 30 cycles: denaturation (96^{o}C/30 sec), aligning (50^{o}C/15 sec) and polymerization (60^{o}C/4 min). PAGE running was done by U. Coja (Spezielle Botanik, University of Osnabrück).

Sequence entry, primary analysis and open reading frames (*orf*) searches were performed using the Vector NTI 6 and the Clone Manager 7 programs. Database searches using the PAM120 scoring matrix were carried out with BLAST algorithms (blastN, blastP and blastX), as implemented on the NCBI file server [blast@ncbi.nlm.nih.gov] (Altschul et al. 1990), including the "*S. coelicolor* Genome Database", http://jiio16.jic.bbsrc.ac.uk/streptomyces/. Multiple sequence alignments were generated by means of the CLUSTAL W (1.60) program (Higgins et al. 1992) and multiple sequence alignment editor GeneDoc (version 2.6.002; Nicholas et al. 1997). Codon usage analysis was done by program FramePlot 3.0beta (Ishikawa and Hotta 1999). Presentation of the protein structure was done by the program Cn3D (version 4.1) from the NCBI (http://www.ncbi.nlm.nih.gov). Prediction analysis of leader peptide sequences was done by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al. 1996 and 1997).

III Results

1. Physiology of fusidic acid biotransformation

S. lividans enzymatically converts fusidic acid into 16ß-hydroxy fusidic acid (16ß-OH), which further undergoes spontaneously to its lactone derivative (von Haar et al. 1991). During prolonged cultivation of *S. lividans* the disappearance of the 16ß-OH and the appearance of several new substances were observed (Fig. 3.1).

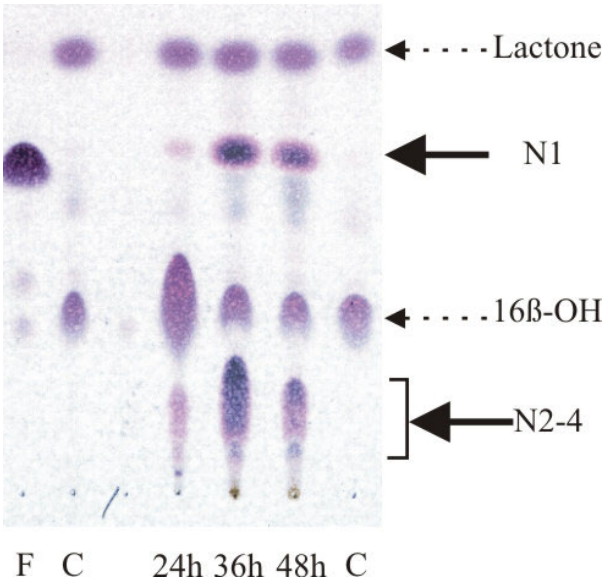


Figure 3.1 In vivo conversion of fusidic acid by *S. lividans*

The strain *S. lividans* was cultivated as follows: Minimal medium supplemented with 1% glucose was inoculated with spores of *S. lividans* and left shaking for 24 h. The culture was then diluted 1:1 with fresh media containing 5 µg/ml fusidic acid and shaking was continued for 12 hours. To the culture fusidic acid was then added (30 µg/ml, starting point-0 h) and the cultivation continued for 48 h. In indicated intervals: 24 h, 36 h and 48 h after the addition of the fusidic acid, aliquots were taken, extracted with ethylacetate and analyzed by TLC. Abbreviations: F- fusidic acid (Rf 0.45) C- 16ß-hydroxy fusidic acid (16ß-OH Rf 0.20) and the lactone derivative (Rf 0.61), N1 to N4 are other substances that appeared during cultivation. Substances N2 to N4 were resolved using 2D- TLC (data not shown).

Within 24 h fusidic acid was completely converted to 16ß-OH, which was gradually converted to the new substances in the later times (36 h and 48 h, Fig 3.1). The lactone derivative and 16ß-OH, when added directly to the liquid culture of *S. lividans* were not converted even after prolonged cultivation (Fig. 3.2).

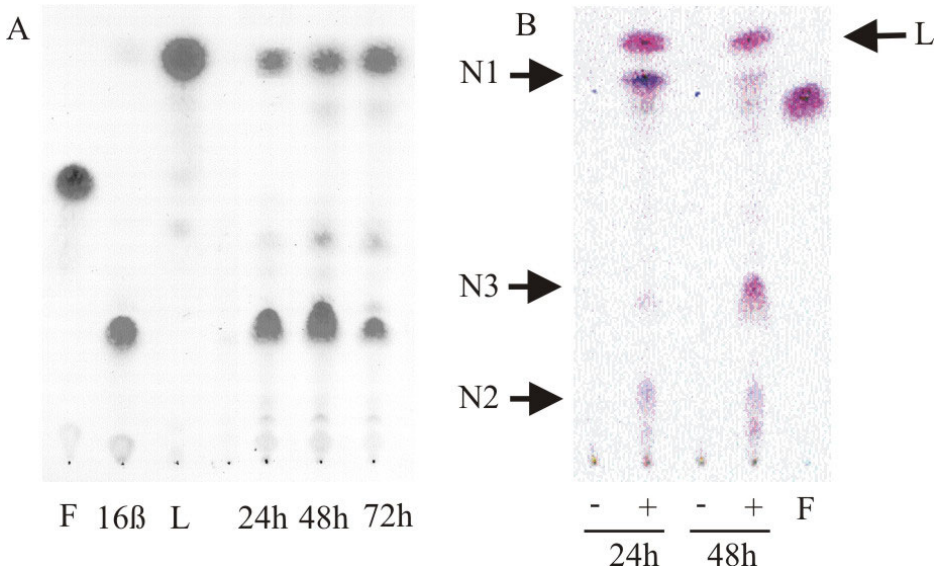


Figure 3.2 In vivo conversion of fusidic acid intermediates

The strain *S. lividans* was cultivated as follows: Minimal medium supplemented with 1% glycerol was inoculated with spores of *S. lividans* and kept standing for 17 hours at 30°C. The culture was then diluted 1:1 with the fresh minimal medium containing either 5 µg/ml fusidic acid (**B**) or none (**A**) and shaking was continued for 24 h. To the culture was then added 30 µg/ml mixture of 16ß-OH and the lactone (starting point, 0 h) and the cultivation continued for up to 72 h. In indicated intervals: 24 h, 48 h and 72 h after addition of the16ß-OH and the lactone, aliquots were taken, extracted with ethylacetate and analyzed by TLC. Abbreviations: F-fusidic acid, 16ß- 16ß-hydroxy fusidic acid, L-lactone, N1-N3 new substances, -/+ addition of the mixture of the lactone and the 16ß-OH to the liquid culture of *S. lividans*.

Upon induction of the strain with the minimal concentration of fusidic acid, the 16ß-OH was completely converted within 24 h, with the appearance of the new substance (N1) (Fig. 3.2B). In order to determine the optimal cultivation conditions for conversion of fusidic acid intermediates, different carbon sources were tested (glucose, glycerol and yeast extract) (Fig. 3.3).

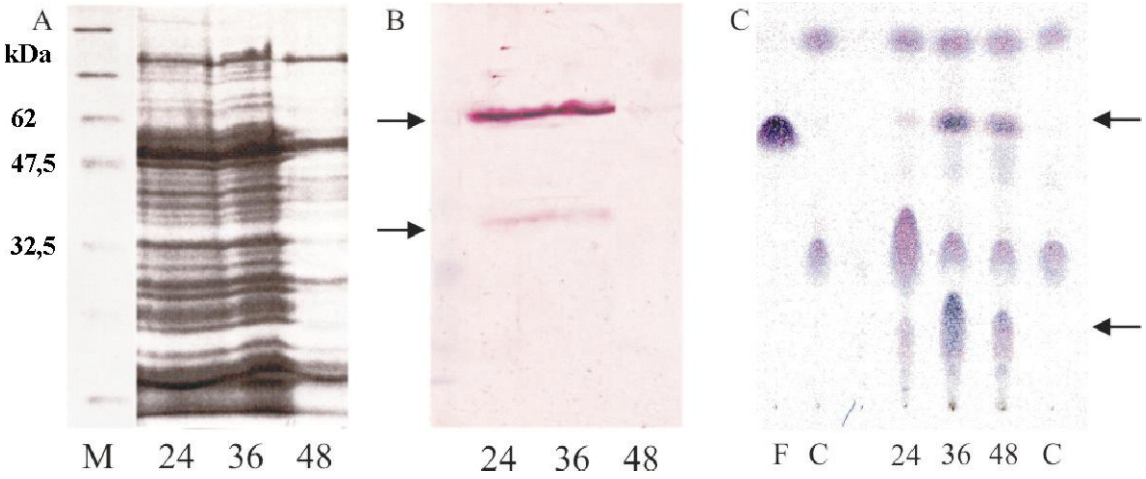
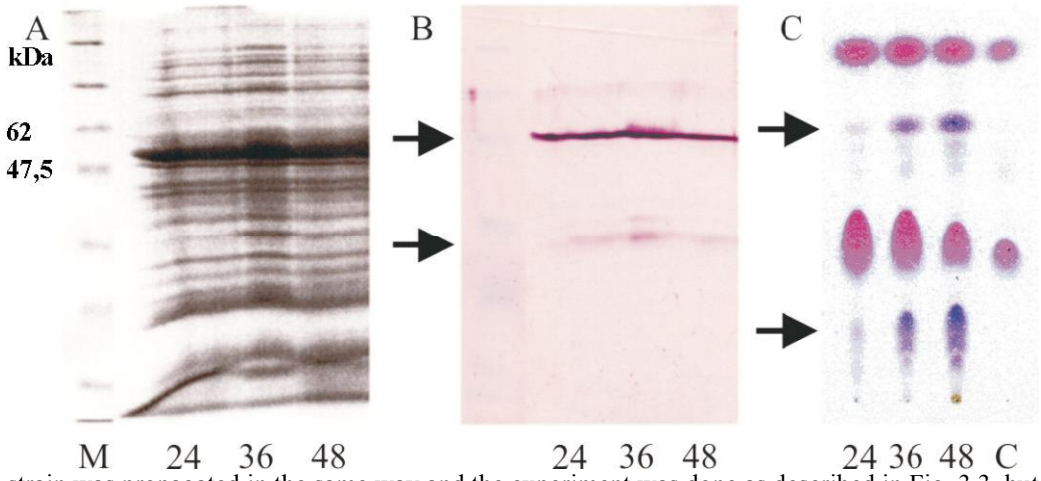


Figure 3.3 Conversion of fusidic acid by *S. lividans* grown in the presence of glucose

Minimal medium supplemented with 1% glucose was inoculated with spores of *S. lividans* and left shaking for 24 h. The culture was diluted with fresh medium 1:1, 5 µg/ml fusidic acid was added and shaking continued for another 12 h. Then the final amount of fusidic acid was added (30 µg/ml) and the propagation continued for defined times: 24 h, 36 h and 48 h. Proteins from the culture filtrates were precipitated by $(NH_4)_2SO_4$ (90% w/v) The presence of FusH (the full length and the truncated form respectively, B- marked by arrows) in isolated protein extracts was immunologically detected by Western blot analysis using the *anti*-FusH antibodies (**B**). In indicated intervals: 24 h, 36 h and 48 h after the addition of the fusidic acid, aliquots of the liquid culture of *S. lividans* were taken, extracted with ethylacetate and analyzed by TLC (**C**).

A-SDS-PAGE analysis of proteins from the culture filtrate. M- protein marker 8S. **B**- Western blot analysis using the *anti*-FusH antibodies. The products 48 kDa FusH and its 35 kDa truncated form are marked by arrows. **C**-TLC analysis of the fusidic acid conversion. New substances Rf 0.5 and Rf 0.1-0.15 are indicated by arrows. F- fusidic acid, C- mixture of the 16ß-OH and the lactone. The numbers represent the times passed (in hours) after addition of 30 µg/ml fusidic acid.

Figure 3.4 Conversion of fusidic acid by *S. lividans* grown in glycerol



The strain was propagated in the same way and the experiment was done as described in Fig. 3.3, but 1% glucose was substituted by 1% glycerol.

A- SDS-PAGE analysis of the precipitated proteins from indicated times (24 h, 36 h and 48 h). M-protein marker 8S. **B**-Immunodetection of FusH among the separated proteins (A) by the Western blot analysis using the *anti*-FusH antibodies. Arrows show a full size of the 48 kDa FusH and its ~35 kDa truncated form. **C**- TLC analysis of in vivo conversion of the fusidic acid during cultivation of *S. lividans*. The arrows show newly synthesized substances. C (the control) is a mixture of the 16ß-OH and the lactone.

As shown, (Fig. 3.3 and 3.4) the major portion of fusidic acid was converted if *S. lividans* was grown for 24 h in minimal media either supplemented with glucose or glycerol. The differences became evident with the conversion of the 16ß-OH. In the media with 1% glucose 16ß-OH was converted faster and the products were accumulated within 36 h. In MM/ 1% glycerol, the conversion was clearly slower and the majority of the products was accumulated later, i.e. at 48 h. (Fig 3.3C and 3.4C). The amount of secreted proteins in the liquid culture of *S. lividans* was the same in first 36 h for both media, and then a significant decrease occurred in the case of MM/ 1% glucose (Fig 3.3). These differences were also reflected by the differences in the secretion of FusH (Fig 3.3 and 3.4B). The precultivation of *S. lividans* (germination of spores and a propagation of the mycelium), in rich medium (containing yeast extract, Fig. 3.5) proved to be better than the minimal media containing either glycerol or glucose (Fig 3.3 and 3.4).

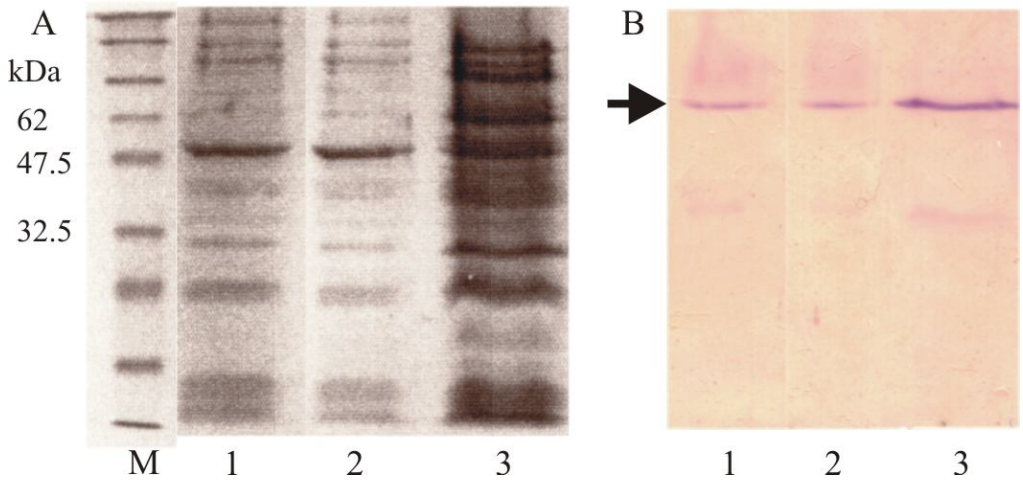


Figure 3.5 Precultivation studies of *S. lividans* in minimal media supplemented with different carbon sources

S. lividans spores were germinated in minimal media supplemented with the 1% glycerol (1), 1% glucose (2) and 1% yeast extract (3) overnight, and after adding 5 µg/ml fusidic acid continued shaking for 24 h. (**A**) The proteins from the culture filtrate were precipitated by $(NH_4)_2SO_4$ (90% w/v) and analyzed by SDS-PAGE. M-protein marker 8S. (**B**) The presence of the 48 kDa FusH was immunologically detected by Western blot analysis using the *anti*-FusH antibodies. The arrow shows detected the FusH enzyme.

Proteins from the culture filtrate of *S. lividans* were concentrated and tested for the enzymatic activity using 16ß-OH and the lactone derivative as substrates (Fig. 3.6).

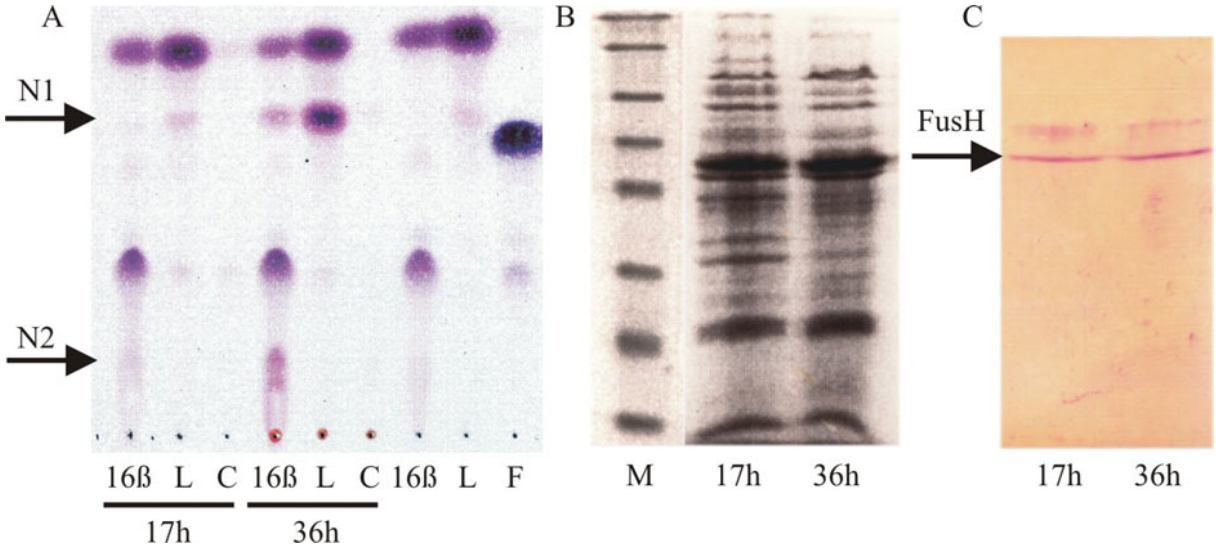


Figure 3.6 Enzymatic conversion of fusidic acid intermediates

Minimal medium containing 0.5% yeast extract was inoculated with spores of *S. lividans*. After germination at 30°C overnight (standing culture) and further shaking for 24 h, the mycelium was washed and shifted to minimal media containing 1% glycerol and 30 µg/ml fusidic acid. The cultivation continued for another 36 h. Aliquots from the culture filtrates were taken at the indicated times (17 h and 36 h), ultraconcentrated (**B**) and used for testing the enzymatic activity, i.e. conversion of 16ß-OH and the lactone derivative respectively. The assay was done in 25 mM Na-citrate buffer pH 5; 2 h/ 45°C and analysed by TLC (**A**). The presence of 48 kDa FusH among the precipitated proteins from the culture filtrate was immunologically detected by Western blot analysis using the *anti*-FusH antibodies (**C**). Abbreviations: 16ß- 16ß-OH fusidic acid, L-lactone, F-fusidic acid, M-protein marker 8S, N1 (Rf 0.50) and N2 (Rf 0.12) are the products of the enzymatic conversion(s); C (control) the assay containing the enzyme extract, but not the substrate.

The enzymes that converted the 16ß-OH and the lactone were produced under the described conditions after prolonged (36 h) incubation (Fig. 3.6A). The lactone was mostly converted into the product N1 (Rf 0.5). The enzyme FusH which converts fusidic acid to 16ß-OH, was present in both tested protein extracts (Fig. 3.6C).

2. Purification of fusidic acid intermediates

Physiological studies revealed the lactone ($C_{29}H_{44}O_4$; MW 456; Rf 0.60) as one of the intermediates that is enzymatically converted by *S. lividans*, most likely, into the two substances A and B with the same Rf 0.5 value. The lactone derivative and both substances A and B were extracted from the culture filtrate and further purified by Flash chromatography and Reversed phase-HPLC (Fig. 3.7).

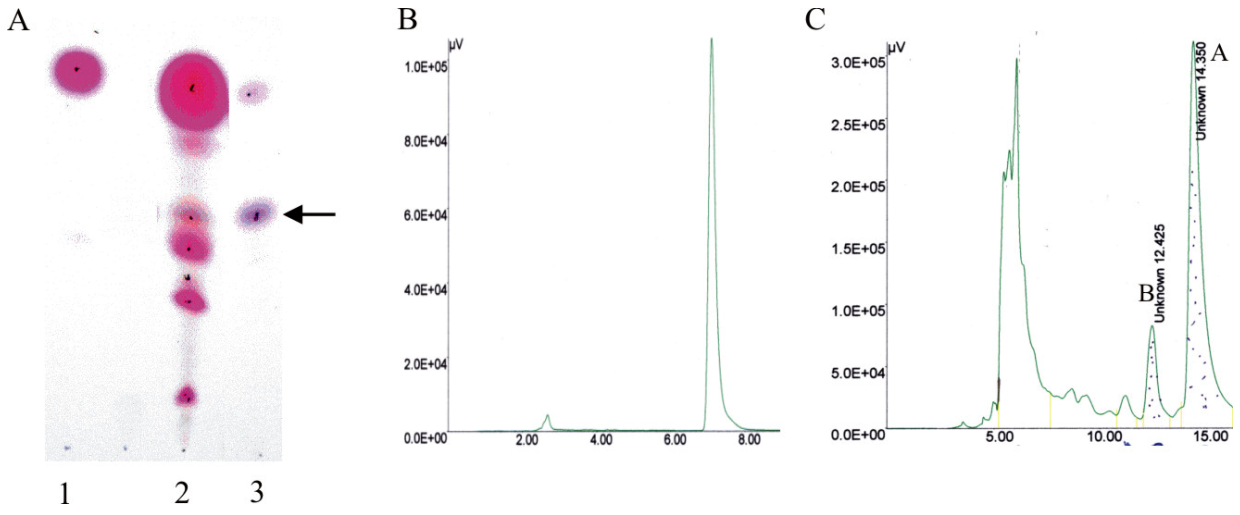


Figure 3.7 Isolation of the fusidic acid intermediates produced by *S. lividans*

The strain was propagated as previously described (see Materials and methods). Fusidic acid intermediates were isolated from the culture filtrate of *S. lividans* by ethylacetate extraction. The organic phase was treated with dry Na_3SO_4 and concentrated by evaporation. The crude concentrate (A-lane 2) (2.89 g) was subjected to Flash chromatography (silica Kieselgur 60 H 60x60 mm, pressure 1 bar), using as eluent dichloromethane: methanol = 97:3. Fractions were analyzed by TLC and were collected in pools 1 to 5. Lactone derivative (A- lane 1 and B; Tr 7.09 min.) was purified from the pool 1 (29.2 mg) and the substances A (C- Tr 14.35 min.; 3.75 mg) and B (C- Tr 12.42; 2 mg) from the pool 2 (97.28 mg), by preparative Reversed phase HPLC (C18, 250x4 mm, 5 µm; Macherei-Nagel) using isocratic elution acetonitril: water (ACN/water) = 80:20 (lactone as shown in B) or 65:35 (substances A and B as shown in Fig. 3.7C).

A-TLC of crude extract of the culture filtrate of *S. lividans* containing lactone (Rf 0.60) and the substances A and B (Rf 0.50), 1- lactone, 2- the crude extract, 3- substances A and B. The arrow shows the substances A and B (Rf 0.5).

B-RP-HPLC Profile of the lactone derivative obtained from the pool 1. Reversed phase C18 HPLC (isocratic gradient ACN/water = 80:20), lactone (Tr 7.09 min.).

C- RP-HPLC Profile of the substances A and B obtained from the pool 2. Reversed phase C18 HPLC (isocratic gradient ACN/water = 65:35), substance A (Tr 14.35 min.) and B (Tr 12.42 min.)

It was found, that the substance A, at elevated temperatures (45°C) spontaneously degraded into several compounds (Fig. 3.8).

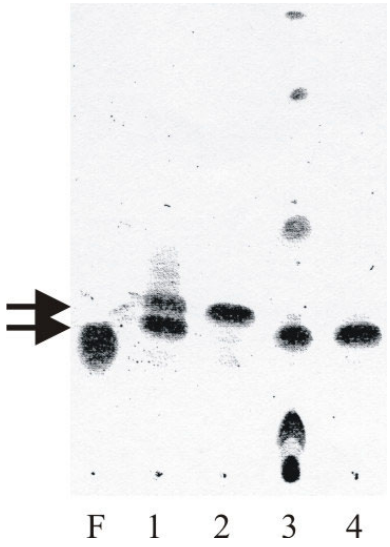


Figure 3.8 TLC analysis of purified substances A and B from the pool 2

Substances B (lane 2) and A (lanes 3 and 4) were separated from the pool 2 (lane 1) on the Reversed phase C18-HPLC (see Fig. 3.7) and concentrated in vacuo by use of the rotary evaporator. During concentration at elevated temperature (45°C) substance A (lane 3) was partially broken down into several substances. F-fusidic acid as a control, 1- pool 2 (purified from the Flash chromatography), 2- substance B, 3- substance A concentrated at 45°C, 4- substance A concentrated at room temperature. The arrows point to the substances A and B.

Pure lactone derivative and substances A and B were subjected to mass spectrometrical analyses (^{13}C and ^{1}H-NMR). The mass spectrum of substance B indicates a protonated molecule $(M+H)^+$at m/z 473, indicating that substance B has a molecular weight of 472. The molecular weight for the substance A was obtained from the mass spectrum, in which the $(M+Na)^+$ ion appears at m/z 495.3. Data for the substance B, as an example are summarized in the Table 2.

B ($C_{29}H_{44}O_5$): ^{1}H-NMR ($CDCl_3$): δ= 1.55, 2.33 (H_2-(C(1)); 1.71, 1.87 (H_2-(C(2)); 4.18 (H-(C(3)); 1.49 (H-(C(4)); 2.57 (H-(C(5)); 1.82, 1.27 (H_2-(C(6)); 1.68, 1,22 (H_2-(C(7)); 1.53 (H-(C(9)); 4.40 (H_2-(C(11)); 2.11, 1.85 (H_2-(C(12)); 3.53 (H-(C(13)); 2.30, 1.25 (H_2-(C(15)); 4.99 (H-(C(16)); 0.82 (H_3-(C(18)); (H_3-(C(19)); 2.24, 2.36 (H_2-(C(22)); 2.22 (H_2-(C(23)); 5.11(H-(C(24)); 1.6 (H_3-(C(26)); 1.68 (H_3-(C(27)); 1.55 (H_3-(C(28)); 2.45 (H-(O(29))
B ($C_{29}H_{44}O_5$): ^{13}C-NMR ($CDCl_3$): δ= 29.91 (C(1)); 30.31 (C(2)); 71.24 (C(3)); 42.27 (C(4)); 31.04 (C(5)); 20.52 (C(6)); 31.43 (C(7)); 40.80 (C(8)); 50.38 (C(9)); 37.02 (C(10)); 67.98 (C(11)); 31.85 (C(12)); 38.09 (C(13)); 55.19 (C(14)); 34.13 (C(15)); 81.82 (C(16)); 169.74 (C(17)); 20.03 (C(18)); 22.99 (C(19)); 123.55 (C(20)); 176.52 (C(21)); 24.06 (C(22)); 27.42 (C(23)); 123.30 (C(24)); 132.78 (C(25)); 17.78 (C(26)); 25.67 (C(27)); 23.08 (C(28));
B ($C_{29}H_{44}O_5$): MS (ESI, 50eV) 473.3 (MH^+); 495.3 (MH^++ Na); 437.3 (MH^+- 36 (H-Cl)); 401.1 (MH^+ - 72.2); 379.3 (MH^+ -94 (71+Na)); 306.1 $(C_{18}H_{25}O_4)^+$ (MH^+ -167.2); 265.1 (MH^+ -208.2)); 233.1 (MH^+ -240.2 (217.2+Na)); 211 (MH^+ -262.3 (240.3+Na)); 183 (MH^+ -290.3); 140.9 (MH^+ -332.4)

Table 2 Selected ^{1}H-NMR, ^{13}C-NMR and MS (ESI, 50eV) data for the substance B ($C_{29}H_{44}O_5$)

Analysis of the collected data and their comparison with the known structure of the lactone derivative ($C_{29}H_{44}O_4$; MW 456; Rf 0.60) (Godtfredsen and Vangedal 1962, von Haar et al. 1991) was done by Dr. H. Kasch and Dr. B. Liedke, (Hans Knöll Institute Jena) and led to the elucidation of the structures of the substances A (Rf 0.50) and B (Rf 0.50) (Fig. 3.9).

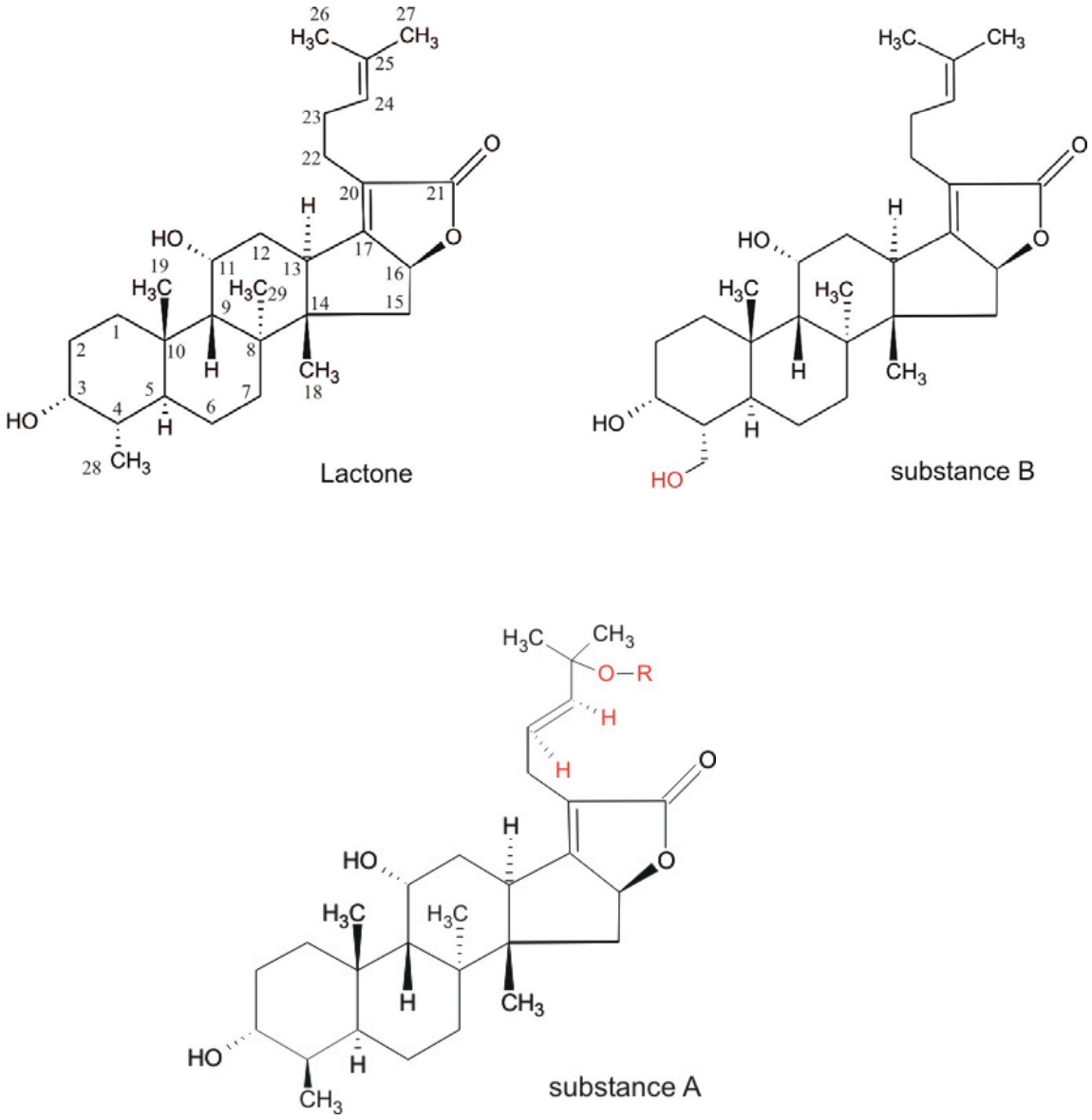


Figure 3.9 Deduced structures of the purified substances A and B in comparison to the lactone

Substance B with the chemical formula $C_{29}H_{44}O_5$ (molecular weight: MW 472) differs from the lactone only in the hydroxyl group present at C-28. The thermolabile substance A, $C_{29}H_{43}O_5$; with the molecular weight of 471 shows the structural difference in the side chain. The double bond C24-C25 present in the lactone is shifted to C23-C24 and on C25 there is additionally attached –O:R as a conjugated base (R is of inorganic origin, likely Na^+ or K^+). The hydrogens on the double bond C23-C24 are in *cis* configuration.

3. Partial purification and characterization of the lactone-converting enzyme FusB

It was tried to biochemically characterize the enzyme (named FusB) that most likely modifies the lactone derivative to the substance N1 (Rf 0.50), (see Fig. 3.6A). For this purpose several different chromatography methods were tested: anion (DEAE) and cation (CMC) exchange chromatography, hydrophobic interaction chromatography (Phenyl sepharose), affinity chromatography (dye ligand), preparative isoelectrofocusing (Rotafor) and Reversed phase chromatography (C4-RP-HPLC).

3.1 Ion exchange chromatography

Since the isoelectric point of FusB enzyme was not known, the binding studies using anion DEAE (diethylaminoethyl) and cation CMC (carboxymethyl cellulose) exchangers were done as a batch experiment. Cation exchanger that binds positively charged proteins was tested in the pH range from 5 to 6.5 (in 0.5 pH increments). Anion exchanger that binds negatively charged proteins was tested in the pH range from 6 to 8 (Fig. 3.10).

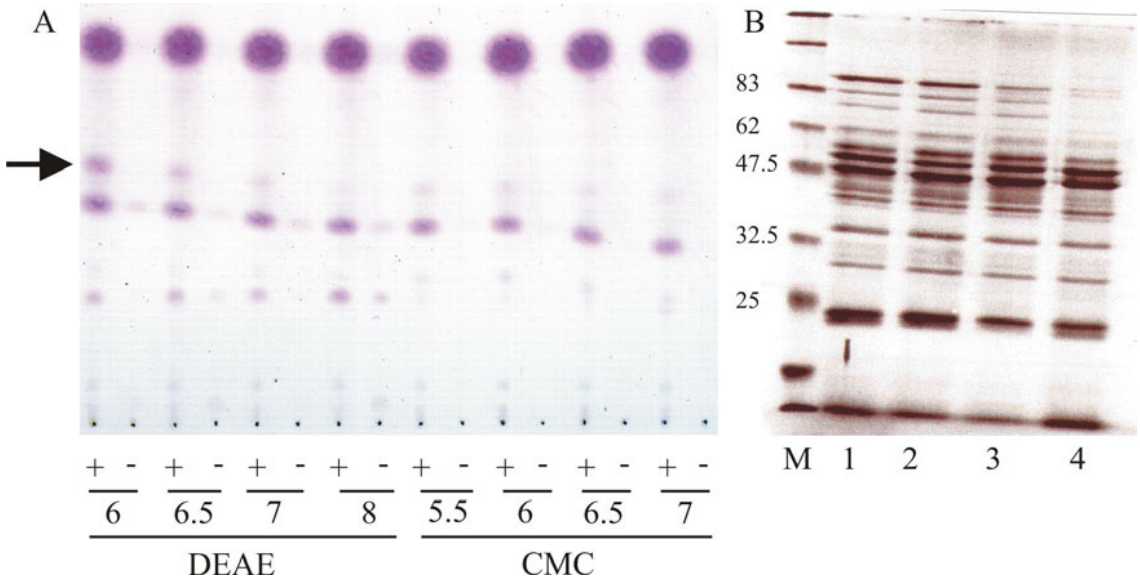


Figure 3.10 Batch binding analysis of FusB using ion exchange matrixes DEAE and CMC.

Streptomyces lividans was grown as previously described (see Materials and methods). After propagation, the culture filtrate was separated from mycelia by centrifugation (20 min., 10^4xg), and further consecutive filtration through paper filter (Schleicher and Schuell), and cellulose acetate filters (1.2 µm and 0.4 µm, Schleicher and Schuell). The pH of the aliquots from the culture filtrates was adjusted and mixed with the previously equilibrated ion exchange matrices. The following buffers for analysis were used: for the anion exchange: 20 mM bis-Tris pH 6 and 6.5, 20 mM Tris-HCl pH 7 and 8, for the cation exchange: 25 mM Na-malonic acid pH 5 and 6, 25 mM Na- phosphate buffer pH 6.5 and 7. After binding, the matrix was washed with the corresponding

binding buffer and the proteins were eluted with the same buffer containing 1 M NaCl. Aliquots of the fractions were tested for the lactone-converting activity (substrate 5 µg lactone in 25 mM Na-citrate pH 5, 200 µM FAD, overnight at 40°C) and analyzed by TLC (**A**) and by gel electrophoresis (**B**).

A- TLC analysis of the enzyme assays (see Material and methods). The numbers show the tested pH, anion ligand DEAE- diethylaminoethyl, cation ligand CMC- carboxymethyl cellulose-. +/- enzyme assays with (+) and without (-) substrate lactone. The arrow shows the product (Rf 0.5) of the enzymatic reaction. The top compound (Rf 0.6) is unconverted substrate lactone.

B-SDS-PAGE of the eluted proteins from the DEAE anion batch analysis. 1-4: pH: 6, 6.5, 7 and 8. M-protein marker 8S.

The analysis (A) showed that FusB does not bind to the cation CMC, but to the anion matrix DEAE, with subsequent eluting at pH 6 and partly at 6.5. The anion exchange DEAE chromatography showed that FusB binds tightly to the matrix and is likely only partially eluted in the presence of 1 M NaCl (Fig. 3.11).

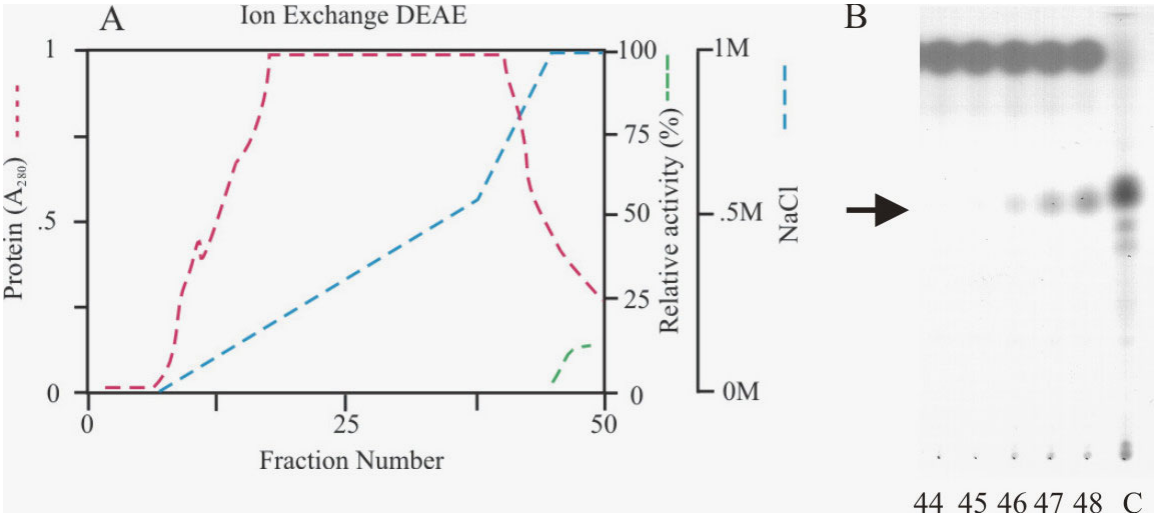


Figure 3.11 The anion DEAE exchange chromatography

Proteins from the culture filtrate, obtained as previously described (see Fig. 3.10) were applied on the DEAE HR5/5 column (**A**) and the bound proteins (red dashed line) were eluted with a linear gradient of NaCl (blue dashed line), 10 column volumes in 20 mM bis-Tris HCl pH 6. Aliquots of eluted fractions were tested for the lactone-converting activity (green dashed line), and the assays were analyzed by TLC (see Materials and methods) (**B**). The enzyme assay was done in 25 mM Na-citrate pH 5, 200 µM FAD/ 40°C overnight. The numbers represent the tested fractions, C- control product Rf 0.5. The arrow shows the product of the enzymatic reaction.

Additionally, it explained why in the binding batch experiment (see Fig. 3.10) with the eluted fractions from the higher pH 7 and 8, the lactone-converting activity was not observed. These results proved the ion exchange chromatography is not suitable to purify FusB.

3.2 Affinity dye ligand chromatography

Dye ligand chromatography separates proteins according to their interactions with the dye ligands, which mimic structurally nucleotide cofactors (NAD; FAD; NADP; ATP). A set of different matrices containing various dyes was used to test the binding affinity for FusB (Fig. 3.12).

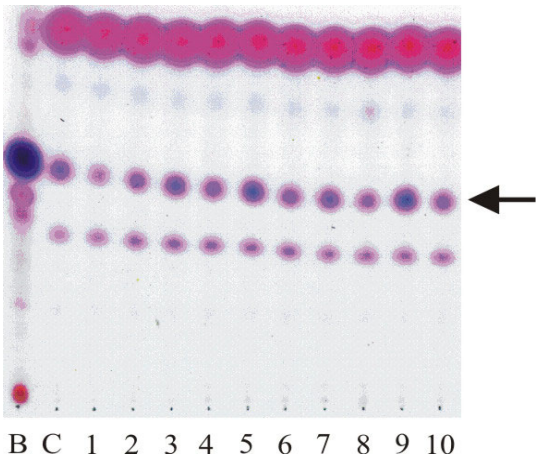


Figure 3.12 Binding analysis of FusB using Affinity dye ligand chromatography

The proteins from the culture filtrate (see Fig. 3.10) of *S. lividans* were aliquoted and were applied to mini columns containing matrices with the different types of the dye ligands (PIKSI Kit, ACL, UK). After excessive washing (5 ml) with 25 mM Na-phosphate buffer pH 6, the remaining bound proteins were eluted with 50mM Na-phosphate buffer pH 8 containing 10 mM FAD. Aliquots of the eluted proteins were assayed for the lactone-converting activity, as described previously (see Fig. 3.10) and the enzyme reactions were analyzed by TLC. Abbreviations: B- product Rf 0.5, C- culture filtrate (control), Mimetic dyes 1-red2, 2-red3, 3-orange1, 4-orange2, 5-orange3, 6-yellow1, 7-yellow2, 8-green1, 9-blue1, 10-blue2. The arrow shows the expected product.

As deduced from the activity tests, FusB binds to the dye ligand matrixes. The way of the interaction with the tested ligands could not be predicted, since the chemical properties of the dyes are not known. For further optimization, blue1 and orange3 matrices were selected (Fig. 3.13).

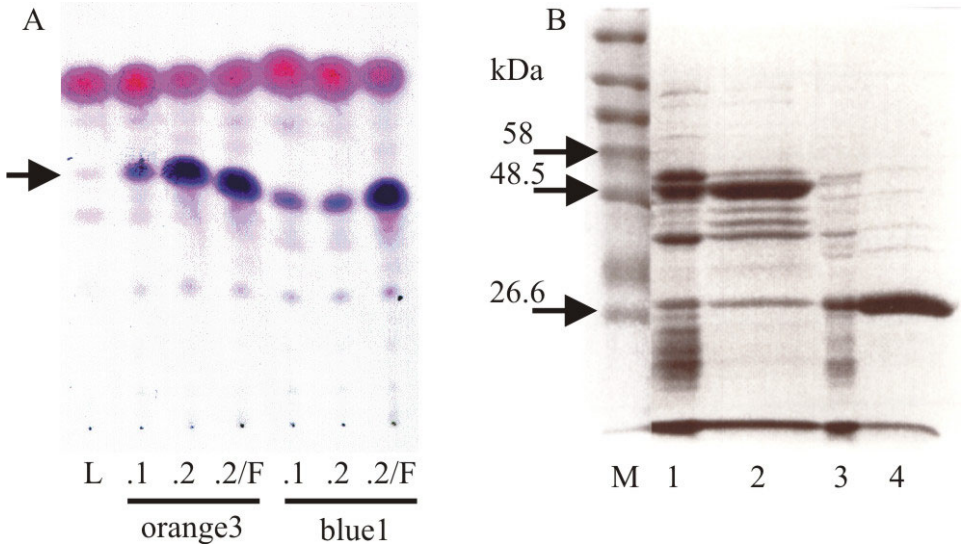


Figure 3.13 Optimization of the conditions for the purification of FusB by Affinity chromatography

S. lividans spores were germinated (30°C) in minimal media (supplemented with 0.5% yeast extract, 5 µg/ml fusidic acid), overnight and then shaken for 15 h. The mycelium was washed and shifted into minimal medium supplemented with 1% glycerol and 30 µg/ml fusidic acid. The culture was further propagated for 29 h.

The culture filtrate (200 ml) was ultraconcentrated to 8 ml and 4 ml were applied to preequlibrated (binding buffer: 25 mM Na-phosphate buffer pH 6) orange3 and blue1 HR 5/5 affinity columns. After excessive washing with the binding buffer, proteins were stepwise eluted with 0.1 M NaCl, (**0.1**) 0.2 M NaCl (**0.2**) and 0.2 M NaCl/ 10 mM FAD (**0.2/F**) in 25 mM Na-phosphate buffer pH 6. Aliquots of eluted proteins were used in enzymatic reactions for converting lactone and for the SDS-PAGE analysis (**B**). The assay was done in 25 mM Na-citrate pH 5, 5 µg lactone, 200 µM FAD/ 40°C, overnight, followed by ethylacetate extraction and the TLC analysis (**A**)

A. TLC analysis of the enzymatic reactions. The top compound is unreacted lactone (Rf 0.6) and the arrow indicates the expected product Rf 0.5. The numbers represent the concentrations of NaCl (0.1 and 0.2 M) used in elution, L-lactone.

B. SDS-PAGE of eluted fractions: 1,2 blue1- 0.2 and 0.2/F, 3,4 orange3- 0.2 and 0.2/F. M-protein marker 7B.

The TLC analysis revealed that FusB binds more strongly on the blue1, then on the orange3 matrix. The majority of the contaminating proteins could be released with the slight increase of the ionic strength of the washing buffer. The fractions eluted with the buffer containing FAD, from the orange3 (B lane 4) and blue1 (B lane 2) have also different protein contents. In the fraction gained from the orange3 (B- lane 4) there is a predominantly ~30 kDa protein. The same protein band is present in the fraction gained from the blue1 (B- lane 2), in which the dominant protein is in the size of ~50 kDa.

3.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography separates proteins according to their differences in hydrophobicity. Partial $(NH_4)_2SO_4$ precipitation of proteins from culture filtrate caused coprecipitation of the fusidic acid intermediates, thereby interfering with the following enzyme assays. Therefore the analysis was done by column Phenyl sepharose chromatography, using the following concentrations of $(NH_4)_2SO_4$: 0.7 M, 1.0 M, 1.3 M, 1.7 M and 2 M (Fig. 3.14).

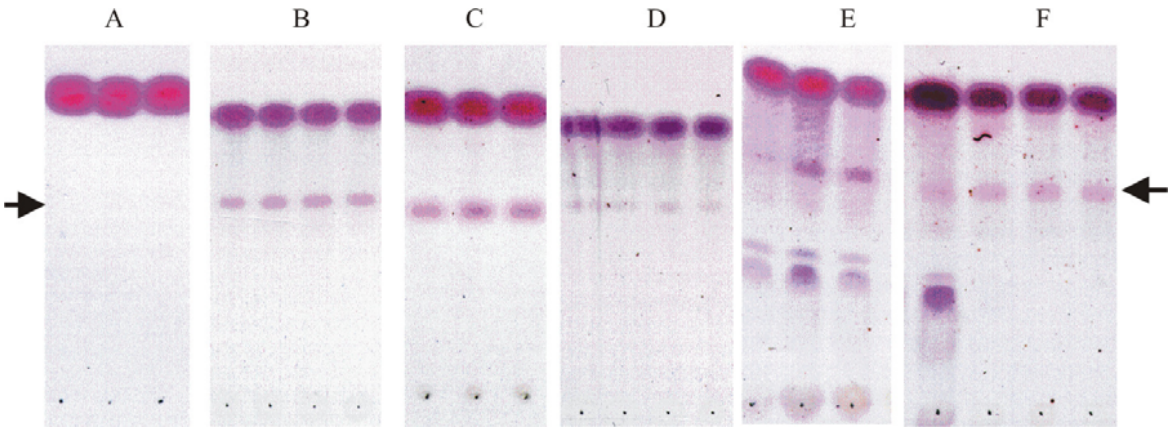


Figure 3.14 Optimization of binding conditions of FusB on Phenyl sepharose matrix

The culture filtrate of the *S. lividans* (see Materials and methods) was aliquoted and to the each $(NH_4)_2SO_4$ was added to the final concentration of 0.5 M (**B**) 0.7 M (**C**), 1 M (**D**) 1.7 M (**E**), 2 M (**F**). In the control (**A**) salt was omited. Cleared filtrates were applied on the previously equilibrated Phenyl sepharose columns (defined concentration of the salt in 25 mM bis-Tris pH 6.5) and the proteins were eluted in 10 column volumes (10 CV). Aliquots of the eluted proteins were tested for the lactone-conversion and the conversions were analyzed by TLC. Active fractions were eluted with the buffer having from 0 to 20% of the starting $(NH_4)_2SO_4$ concentration The arrows marked the expected product (Rf 0.5) of the lactone-conversion.

The analysis showed that FusB binds tightly to the Phenyl sepharose matrix. The optimal concentration of the $(NH_4)_2SO_4$ needed for binding is rather low (0.7 M lane C). The activity obtained using higher $(NH_4)_2SO_4$ concentrations (lane E and F) could be due to the precipitation of the protein on the column and subsequent release in the presence of low salt buffer during elution. The active fractions obtained from the Phenyl sepharose hydrophobic interaction chromatography (0.7 M $(NH_4)_2SO_4$) were used for further purification of FusB (Fig 3.15).

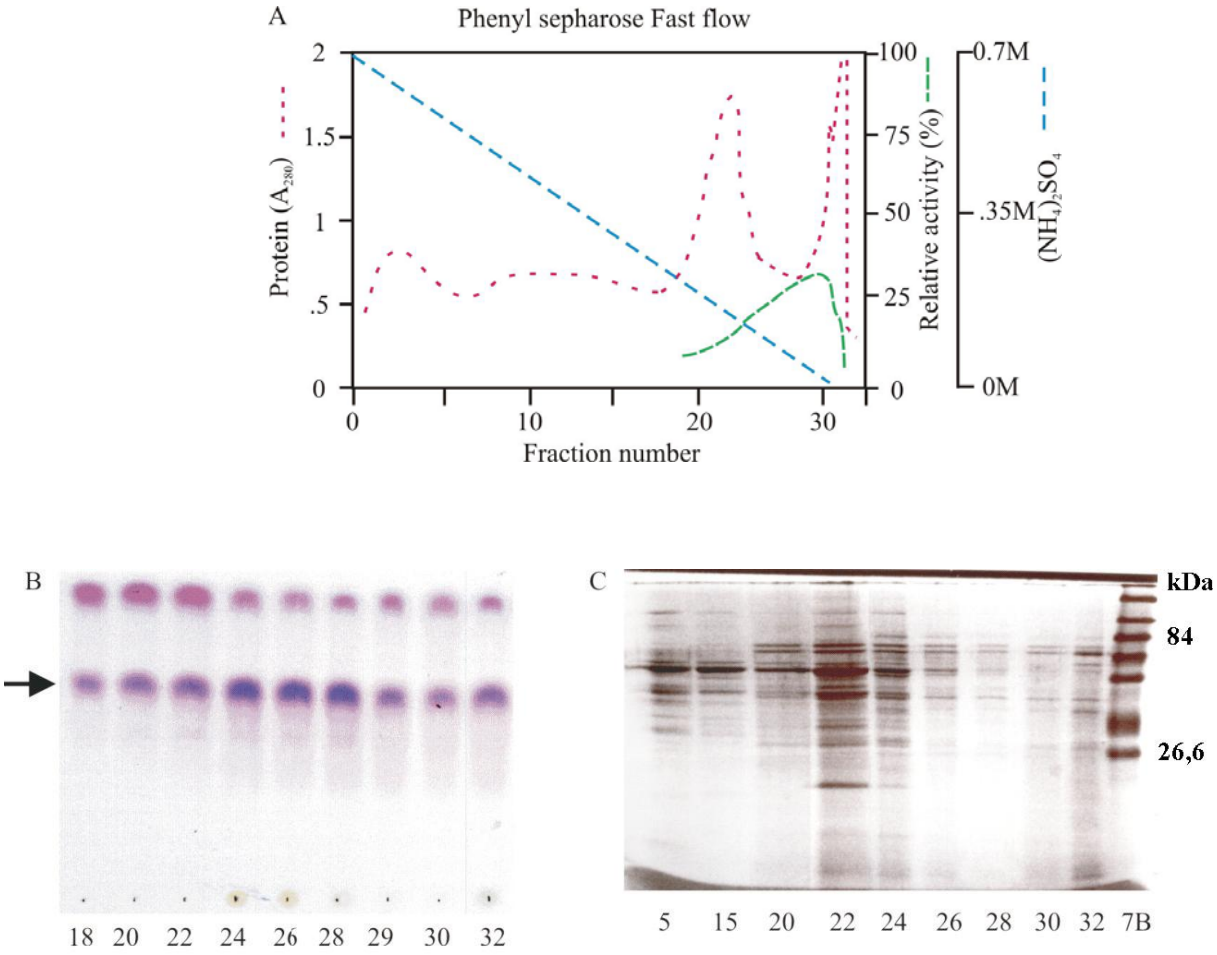


Figure 3.15 Phenyl sepharose hydrophobic interaction chromatography

S. lividans was grown as described previously (see Materials and methods). To the culture filtrate (3 L, 295 mg) the salt was added to a final concentration of 0.7 M $(NH_4)_2SO_4$ and the cleared filtrate was applied to the Phenyl sepharose Fast flow HR 16/20 (**A**). The bound proteins (red dashed line) were eluted with a descending linear gradient from 100-0% $(NH_4)_2SO_4$ (blue dashed line), 6 column volumes (CV) in 25 mM bis-Tris- HCl pH 6.5. Aliquots of eluted fractions were tested for the lactone-converting activity (green dashed line), and the assays were analyzed by TLC. The assay was done in 25 mM Na-citrate pH 5, 5 µg lactone, 200 µM FAD/ 40°C, overnight

B- TLC analysis of enzymatic conversion of the lactone, using isolated protein fractions as enzymes. The arrow shows obtained product Rf 0.5.

C- SDS-PAGE, the numbers from 18 to 32 represent the fractions (volume 6 ml). 7B-protein marker.

Active fractions from 25 to 32 (Fig. 3.15C) were collected, and the proteins were further separated using Affinity blue1 chromatography (Fig. 3.16).

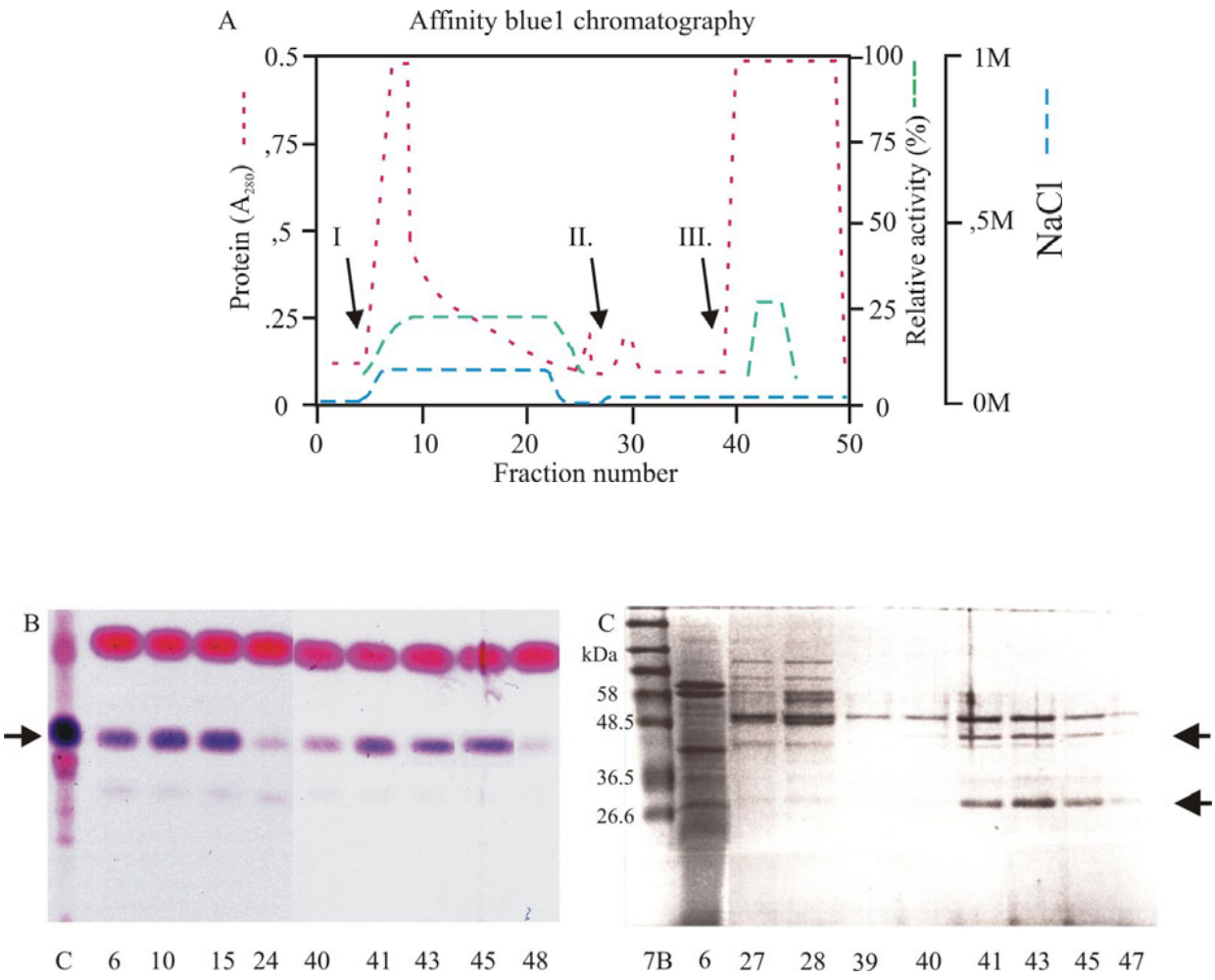


Figure 3.16 Affinity blue1 chromatography

Pool containing fractions between 25/32 from the HIC Phenyl sepharose (see Fig. 3.15 B and C) was dialyzed against 25 mM Na-phosphate buffer pH 6 and applied on the Affinity blue1 chromatography HR5/5 (**A**). The column was excessively washed with the 100 mM NaCl in 25 mM Na-phosphate pH 6 (**I**), and then with the 50 mM Na-phosphate buffer pH 8 (**II**.). The remaining proteins (red dashed line) were eluted with the 5 ml 5 mM FAD in 50 mM Na-phosphate buffer pH 8 (**III**.). Aliquots of separated protein fractions (500 µl) were analyzed by SDS-PAGE (**C**) and for the lactone-converting activity (green dashed line) (**B**). The assay was done in 25 mM Na-citrate pH 5, 5 µg lactone, 200 µM FAD/ 40°C, overnight.

B- TLC analysis of the lactone-conversion by the separated protein fractions from blue1. C indicates standard sample. The arrow shows obtained product Rf 0.5.

C- SDS-PAGE analysis, 7B-protein marker, the numbers represent the isolated fractions (fraction volume 500 µl). The arrows assigned proteins in size of 40 kDa and 30 kDa in the active fractions 41/45 that are potential candidates for FusB.

Active fractions 41/45, which were eluted with the buffer containing FAD have only a few proteins in the size of ~30 kDa, ~40 kDa and ~50 kDa. The same active fractions from the

HIC Phenyl sepharose (Fig. 3.15) were applied on the Affinity orange3 chromatography after dialyzing (Fig. 3.17).

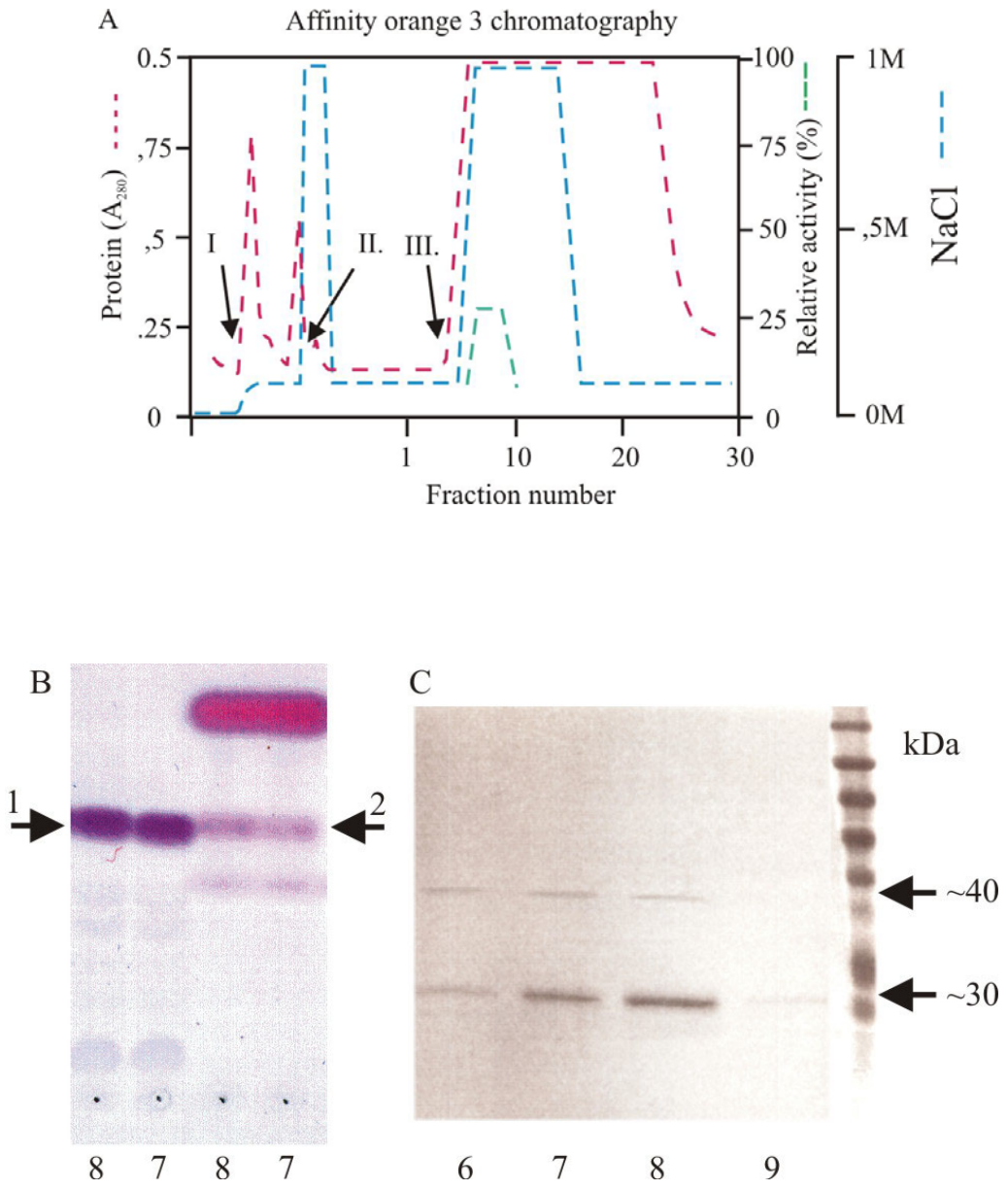


Figure 3.17 Affinity orange3 chromatography

Pool containing fractions between 25/32 from the HIC Phenyl sepharose (see Fig. 3.15B and C) was dialyzed against 20 mM piperazine-HCl pH 5.5 and applied on the Affinity orange3 chromatography HR 5/5 (**A**). The piperazine buffer was replaced by 60 mM Na-phosphate buffer pH 8 (**I**), thereby the weakly bound proteins (red dashed line) were released by increasing the ionic strength and the pH. Then the matrix was once more shortly washed with injecting the 1 ml 1 M NaCl (blue dashed line) (**II**). The remaining bound proteins were eluted with the 5 ml 5 mM FAD, 1 M NaCl in 60 mM Na-phosphate buffer pH8 (**III**). Aliquots of separated proteins released with FAD (500 µl) were analyzed by SDS-PAGE (**C**) and for the lactone-converting activity (green dashed line) (**B**). The assay was done in 25 mM citrate pH 5, 5 µg lactone, 200 µM FAD/ 40°C, overnight

B- TLC analysis of enzymatic conversion of the lactone. 7 and 8 are the tested fractions. 1-fusidic acid, 2- new substance Rf 0.5

C- SDS-PAGE analysis: The numbers represent the isolated fractions (fraction volume 500 µl). Marker (last lane from the left) is the 7B protein standard. Arrows assigned protein bands in the size of ~30 kDa and ~40 kDa that are potential candidates for FusB.

The purification procedure using Affinity orange3 instead of blue1 chromatography obtained enzymatically active fractions containing 2 proteins of ~30 kDa and ~40 kDa. The proteins with the similar size were also obtained from the active protein fractions using blue1 (see Fig 3.16C). Such partially purified FusB was used for further biochemical characterization.

3.4 Biochemical characterization of FusB

Partially purified FusB was used to determine the temperature-, and pH-optima for the lactone conversion (Fig. 3.18).

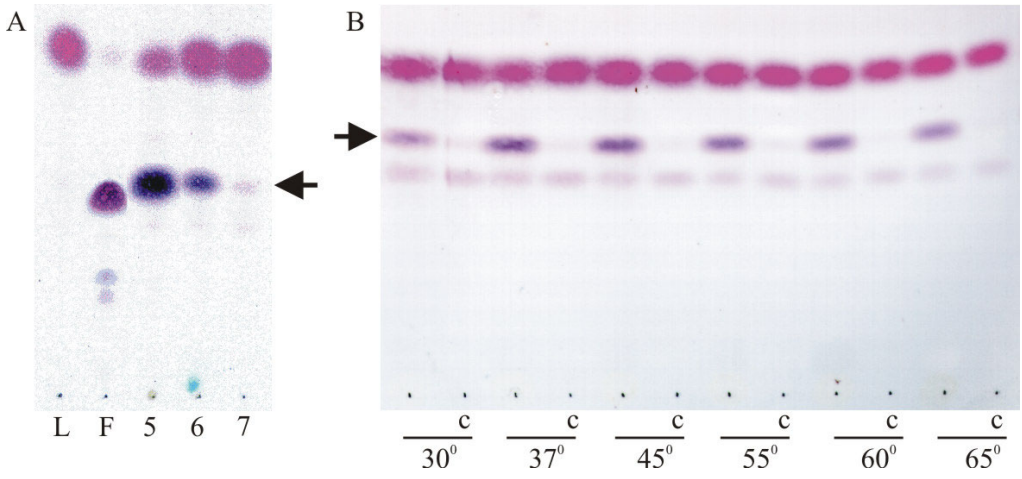


Figure 3.18 Determination of pH- and the temperature-optima for the lactone conversion

A Conversion of the lactone at different pH (5 to 7) by partially purified FusB. Reaction assay was done 8 h/ 45°C at indicated pH: pH 5 (25 mM Na-citrate), pH 6, and pH 7 (50 mM Na-phosphate buffer). Lactone conversion was analyzed by TLC: L-lactone F- fusidic acid, the numbers represent the tested pH. Arrow shows the obtained product Rf 0.5.

B Conversion of the lactone at different temperatures by partially purified FusB. Reaction assay was done overnight at indicated temperatures (30, 37, 45, 55, 60 and 65°C), in 25 mM Na-citrate pH 5, containing 5 µg lactone and 1 mM FAD. Lactone conversion was analyzed by TLC. In figure B the control reaction (C) was done without the enzyme. The arrow shows the expected product Rf 0.5.

The conversion of the lactone is more sensitive to pH, rather than to temperature changes. The reaction occurred between 30°C and 65°C (B), with the highest value between 37 and 45°C. In the tested pH range from 5 to 7 (A), the highest activity was obtained at pH 5. With the increase of pH, the activity of the enzyme decreased rapidly. Enzymatic conversion of the lactone also depends on the cofactor used (Fig 3.19).

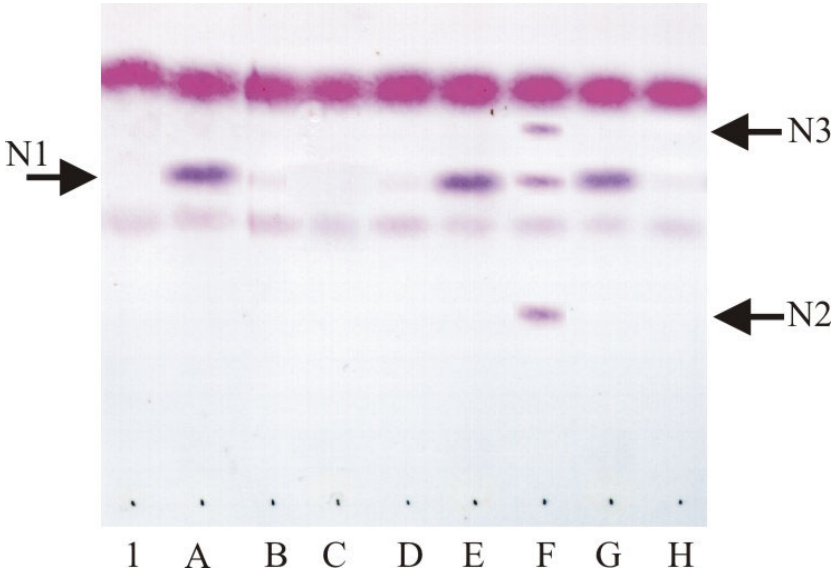


Figure 3.19 Conversion of the lactone in the presence of the cofactors

Test of the partially purified FusB for the lactone conversion in the presence of different nucleotide cofactors used in final concentration of 1 mM: **A**. FAD; **B**. NADP; **C**. NADPH; **D**. ATP+5 mM $MgSO_4$; **E**. FAD/NADP; **F**. FAD/NADPH; **G**. FAD/ATP+5 mM $MgSO_4$ **H**. NADP/ATP+5 mM $MgSO_4$. **1**-reaction without cofactor. Reaction assay was 25 mM Na-citrate pH5, 5 µg lactone overnight/ 40°C. The enzyme assays were analyzed by TLC. Arrows show obtained products: N1- Rf 0.50, N2- Rf 0.32 and N3- Rf 0.57.

The lactone was converted into a substance N1 (Rf 0.50) only in the presence of the cofactor FAD (A). When NADPH together with FAD was used, two new substances N2 (Rf 0.32) and N3 (Rf 0.57) were observed (F). From these results (Fig. 3.19 lanes A, C and F), it could be concluded that partially purified fractions of FusB catalyzed a two step enzyme conversion: from the lactone (Rf 0.6) to the substance N1 (Rf 0.50) by FAD. The second reaction occurred in the presence of NADPH, resulting in conversion of the N1 into two products N2 (Rf 0.32) and N3 (Rf 0.57). Partially purified FusB (Fig. 3.20A), which was obtained by Affinity blue1 chromatography was subjected to Native isoelectrofocusing (IEF, pH gradient 3 to 10). Separated proteins (B and C) were assayed for the lactone-converting activity, and TLC analysis revealed that FusB is an acidic protein with a rather low pI of about 3.8 (Fig. 3.20).

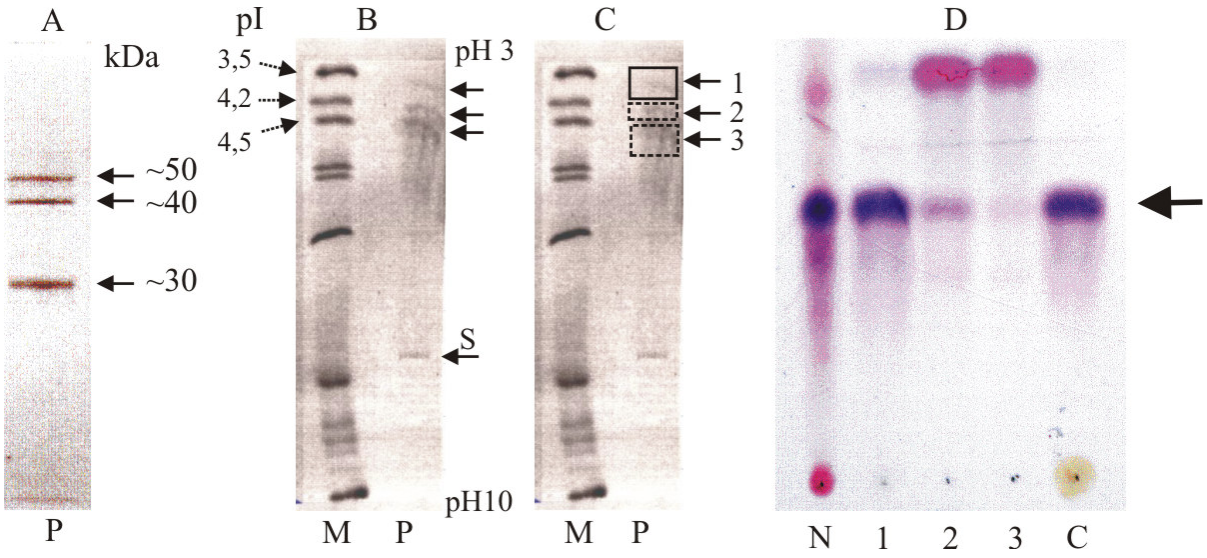


Figure 3.20 Native isoelectrofocusing (IEF) of the partially purified FusB

Partially purified FusB (**A**) was subjected to the native isoelectrofocusing (**B**) in the pH gradient from 3 to 10. Separated protein bands were cut out (**C**) and used for the enzymatic conversion of the lactone. The assays were then extracted and analyzed by TLC (**D**).

A- SDS-PAGE of the partially purified FusB. The numbers represent the sizes of the separated protein bands.

B- Native isoelectrofocusing pH 3-10. Program: **1**. 15 min.-200 V-3 mA-6 W; **2**. 2 h and 15 min.-2000 V-3 mA-6 W. Numbers: 3.5 4.2 and 4.5 are the pI of the marker proteins. The arrows show the separated protein bands. S is a starting point of isoelectrofocusing.

C- The proteins which were previously separated by IEF (shown in B) were cut out of the gel (1, 2 and 3) and used in enzyme reaction. The assay was done in 25 mM Na-citrate pH 5, 5 µg lactone, 100 µM FAD; 40^{o}C/ overnight

D- TLC analysis of the enzymatic conversion of the lactone with the separated proteins. The numbers indicate the cut protein bands used in the assays. N substance Rf 0.5 C- control reaction of the partially purified FusB.

This analysis also confirmed the previous results that the lactone is enzymatically converted to a substance with Rf 0.5 (D, assigned with the arrow).

4. Purification and partial characterization of the enzyme FusG

During purification of FusB, a ~30 kDa protein appeared in the enzymatically active fractions obtained from the Affinity blue1 (see Fig. 3.16C) or the orange3 chromatography column (Fig 3.17C). In gel filtration 30 kDa and 40 kDa proteins migrate together (Fig. 3.21).

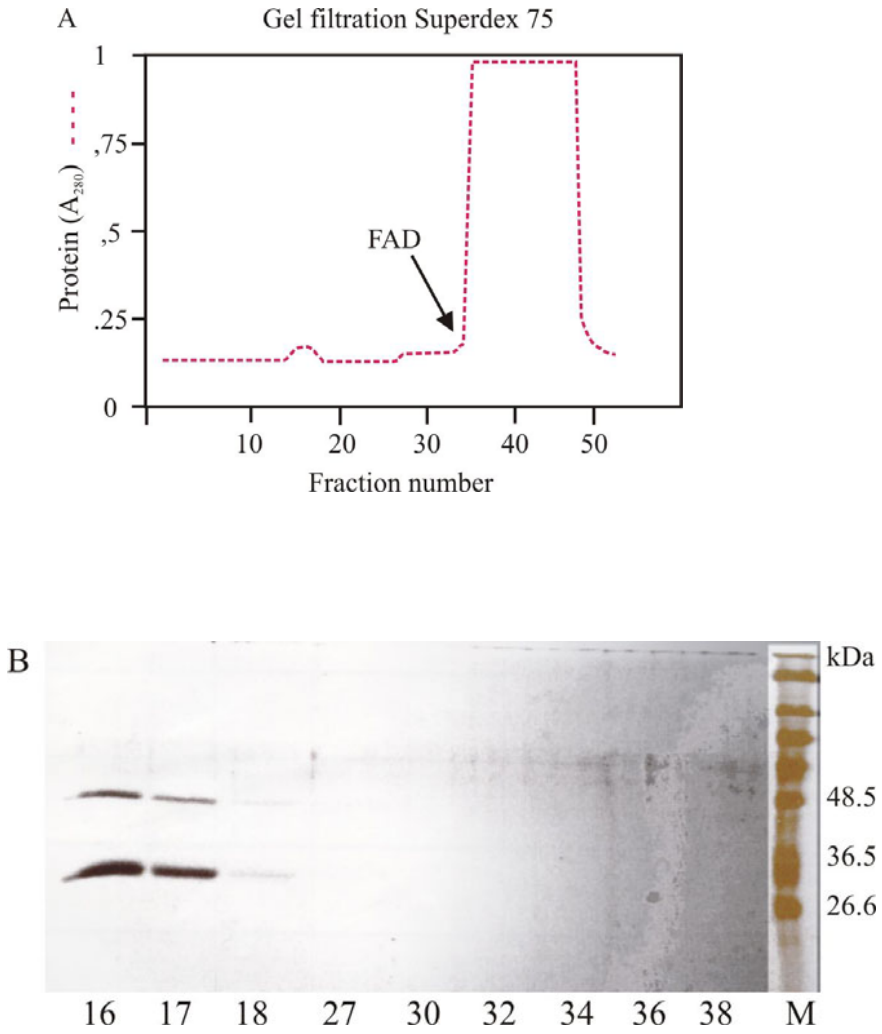


Figure 3.21 Gel filtration Superdex 75 of 30 kDa and 40 kDa proteins

Concentrated sample of partially purified FusB (Fig 3.17C) was applied on Gel filtration Superdex 75 and the proteins were eluted in 25 mM Na-phosphate buffer pH 6, 150 mM NaCl at flow rate of 0.8 ml/ min. (**A**). Arrow indicates on the chromatograph elution of FAD which was present in the probe.

B- SDS-PAGE analysis of the separated fractions from the Superdex 75. M-7B protein marker.

The 30 kDa protein binds to the matrix orange3 more tightly than the 40 kDa protein. This property was used to separate the 30 kDa protein (named FusG) from the rest of the contaminating proteins including the 40 kDa one (Fig. 3.22).

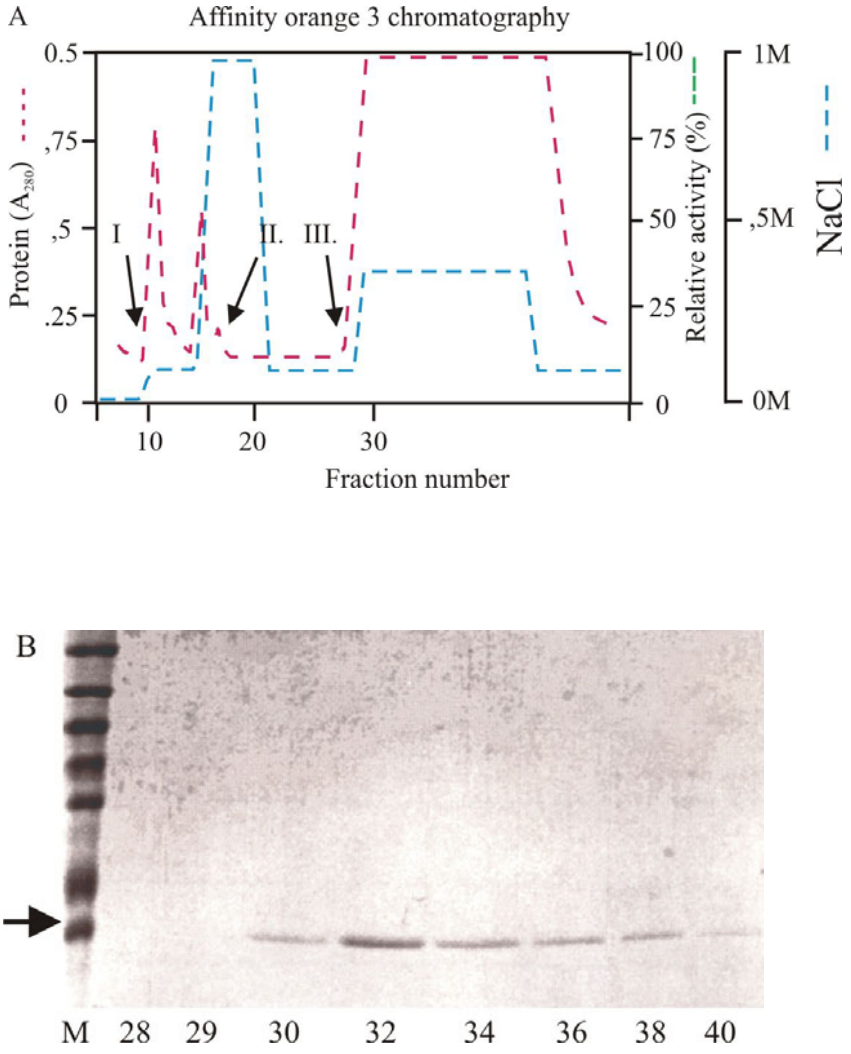


Figure 3.22 Affinity orange3 chromatography

A pool containing fractions between 25/32 from the HIC Phenyl sepharose (see Fig. 3.15) was dialyzed against 20 mM piperazine-HCl pH 5.5 and applied on the Affinity orange3 chromatography HR 5/5 (**A**). The piperazine buffer was replaced by 60 mM Na-phosphate buffer pH 8 (**I**), thereby the weakly bound proteins (red dashed line) were released by increasing the ionic strength and the pH. Then the matrix was once more washed with 5 ml 1 M NaCl (5 CV, blue dashed line) (**II**). The remaining bound 30 kDa (FusG) was eluted with the 5 ml 5 mM FAD, 300 mM NaCl in 60 mM Na-phosphate buffer pH 8 (**III**). Aliquots of the FAD fractions (500 µl) were analyzed by SDS-PAGE.

B- SDS-PAGE analysis The numbers represent the isolated fractions (fraction volume 500 µl). M-protein marker 7B. The arrow shows a ~30 kDa protein band.

Purified FusG protein (30 kDa) was tested for the conversion of the fusidic acid and the lactone derivative (Fig. 3.23).

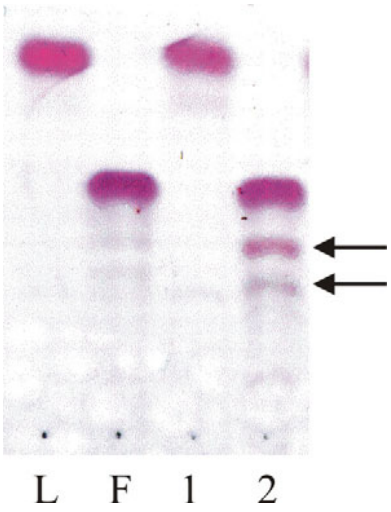
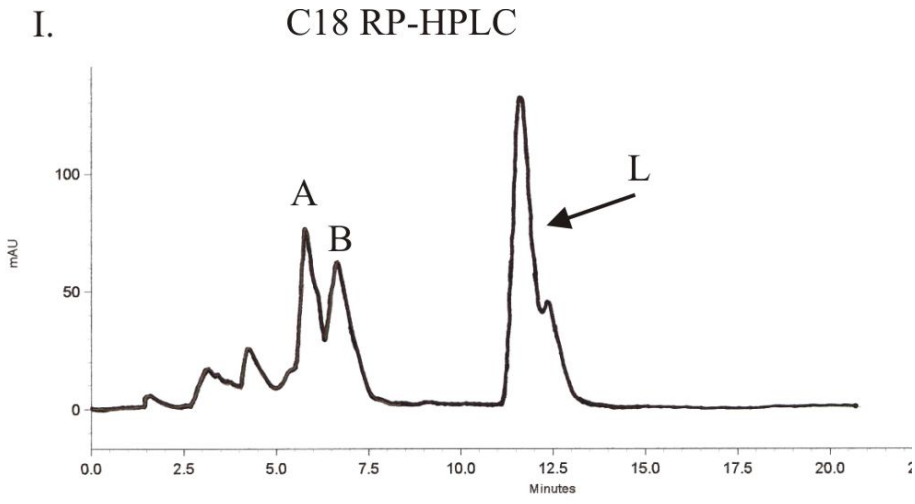


Figure 3.23 Enzymatic conversions of fusidic acid and the lactone by FusG.

The assay was done in 25 mM Na-citrate pH 5 suplemented with 5 µg of the substrate (fusidic acid or lactone) 20 µg bovine serum albumin, 500 µM FAD and NADPH. The reaction was done overnight/ 40°C, further extracted and analyzed by TLC. Abbreviations: L- lactone, F- fusidic acid, 1 and 2 are the enzymatic reactions. The arrows show the detected substances (Rf 0.3 and 0.24) derived enzymatically from the fusidic acid.

The enzyme FusG did not convert the lactone derivative under the described conditions. Using fusidic acid as a substrate, a weak conversion (showed by arrows) to substances with Rf 0.3 and 0.24 was observed. A similar result was obtained with FusG-His tag isolated from the culture filtrate of *S. lividans* (Fig 3.62). A fraction containing the 30 kDa and 40 kDa proteins (see Fig. 3.19, lane F) converted lactone to three substances N1 (Rf 0.5), N2 (Rf 0.32) and N3 (Rf 0.57) in the presence of FAD/NADPH. That experiment confirmed that the N2 and the N3 originated from the N1 substance. Purification of the N1 (Rf 0.50) substance by Reversed phase HPLC revealed that it consists of the two substances with similar Rf value (named A and B) (Fig. 3.24).



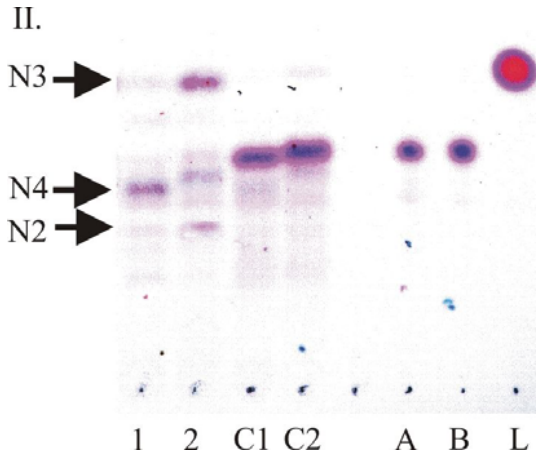


Figure 3.24 In vitro conversion of the purified substances A and B by the enzyme FusG

Lactone has been enzymatically converted by partially purified FusB in to the substances A and B. The reaction was done in 25 mM Na-citrate buffer supplemented with 1 mM FAD and 100 µg of the lactone, 40°C/ overnight. The reaction mix was extracted and the products A (Tr 5.80 min.) and B (Tr 6.68 min.) were separated from the unreacted lactone (Tr 11.60 min.) on Reversed phase HPLC (HyPurity advance 3 µm, column 150x4 mm) with isocratic elution of the methanol:water = 65:35. (**I**.) Separated substances were tested in enzyme reaction using the enzyme FusG (see Fig. 3.22B). The assays were done in 25 mM Na-citrate pH 5 supplemented with 200 µM NADPH, 40°C/overnight and after extraction with ethylacetate analyzed by TLC (**II**).

II.TLC analysis of the assays: 1 and C1-reaction assay with substance A; 2 and C2-reaction with substance B. In C1 and C2 the enzyme FusG was omitted. L- lactone, N2-4 are the products of the enzyme reactions: N2 (Rf 0.32), N3 (Rf 0.57) and N4 (Rf 0.40).

The enzyme FusG is involved in the catalysis (see Fig. 3.19) of the two step-conversion of the lactone via the substances A and B (Rf 0.5, formerly substance N1) to the products N2, N3 and N4, using cofactor NADPH. Thereby the product N4 (Rf 0.40) was derived from the substance A and the products N2 (Rf 0.32) and N3 (Rf 0.57) were derived from the substance B. Both reactions are catalyzed by FusG in the presence of the cofactor NADPH (Fig. 3.24-II). Immunological studies revealed that FusG and FusB are not related to the previously characterized enzyme esterase FusH (von Haar et al. 1997) (Fig 3.25).

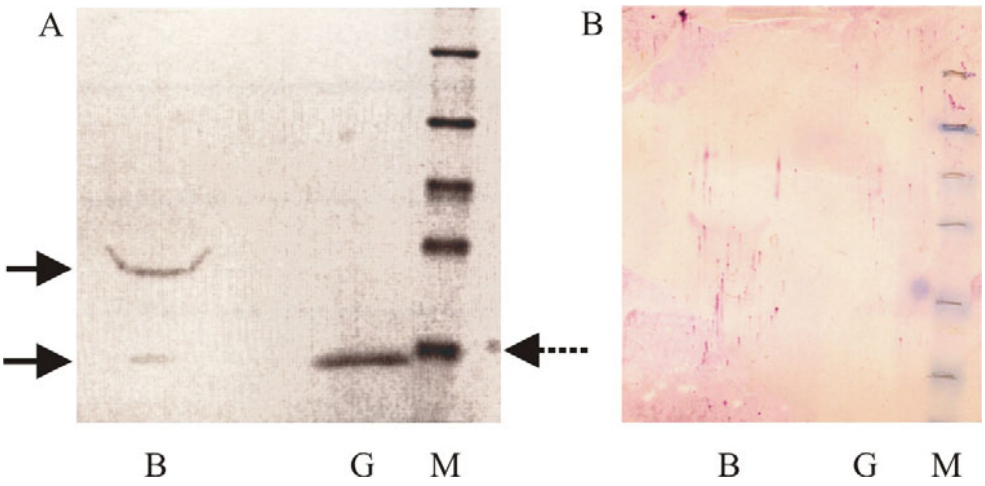


Figure 3.25 Immunodetection of FusH

A- SDS PAGE M- protein marker 8S, G-FusG, B-mixture of FusB (signed B) and FusG (signed G), **B**-Western blot using the *anti*-FusH antibodies. The arrows in A show 40 kDa and 30 kDa proteins.

The enzyme FusG was further subjected to the NH_2-terminal sequencing to subsequently identify the corresponding gene (see Fig. 3.32).

5. Purification and characterization of FusK

5.1 Purification of enzyme FusK

The Ni-NTA enriched fraction of FusA* (protein FusA having 6 histidines on the C-terminus) gained from *S. lividans*, converted the fusidic acid into substances with Rf 0.24 and Rf 0.38. Control purification from *S. lividans* pWHM3 (the strain without FusA*) gave a negative result (see Fig. 3.53). The SDS-PAGE analysis showed that except the cloned FusA* there are few more bands that are present in *S. lividans* pWFA1 transformants, but missing in the control strain *S. lividans* pWHM3. The Ni-NTA binding assay showed that these proteins bound weakly and their binding is inhibited in the presence of 15 mM imidazole. In order to reduce the proteins that unspecifically bind to the matrix, Ni-NTA was previously saturated with FusA* (Fig. 3.26).

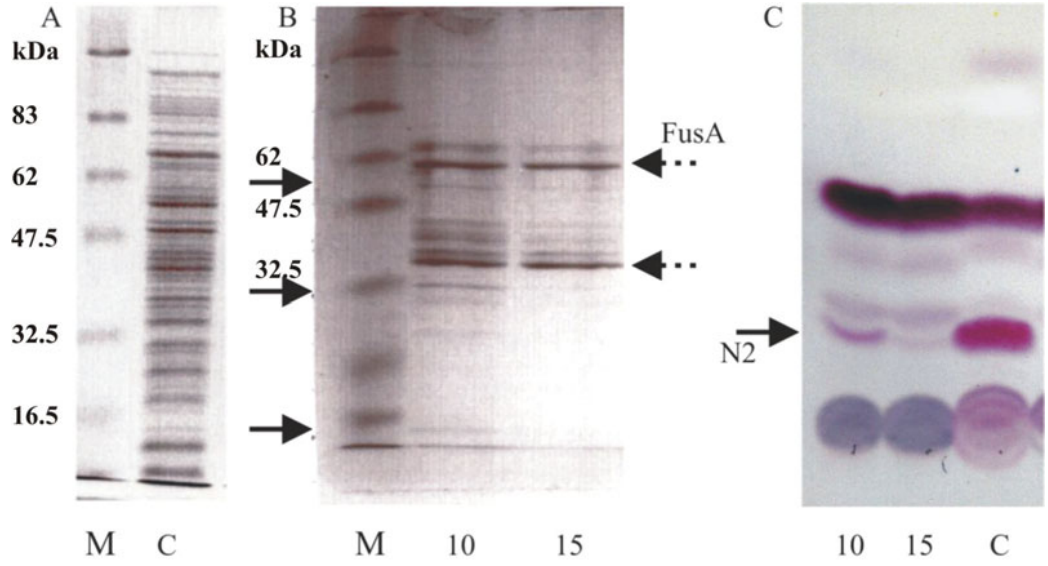


Figure 3.26 The Ni-NTA binding assay, with the matrix previously saturated with FusA*

The cleared supernatant (**A**, sample C), obtained after sonication and centrifugation of mycelia (see Materials and methods) of *S. lividans* pWFA1 transformant was bound on the Ni-NTA-matrix previously saturated with FusA* in the presence of 10 mM or 15 mM imidazole (**B**). After washing with the same imidazole concentration, the matrix was directly used for enzyme reactions and the SDS-PAGE analysis. **B**-SDS-PAGE of 10 and 15 mM represent the fractions, acquired after binding in the presence of assigned imidazole concentrations. FusA* is assigned with shadowed arrows. Full line arrows show the bands (~50 kDa, 32k Da and 15 kDa) that are present in 10 mM fraction but not, or weakly in 15 mM fraction. M- 8S protein marker.

C- TLC analysis of enzyme reactions from obtained fractions (showed in 1A and B). Reaction assays were done in 50 mM na-phosphate pH 7 containing 5 µg fusidic acid for 2 h/ 37^{o}C. Reaction mixtures were extracted (see Materials and methods) and analyzed by TLC. Arrow shows the expected product N2 (Rf 0.24). C- control cleared supernatant obtained after sonication of mycelia.

The enzyme activity test (Fig. 3.26C) revealed that the largest portion of protein(s) responsible for conversion of fusidic acid mostly do not bind to the Ni-NTA matrix. Therefore, proteins which did not bind to the Ni-NTA were further used for purification by hydrophobic interaction chromatography (Fig. 3.27).

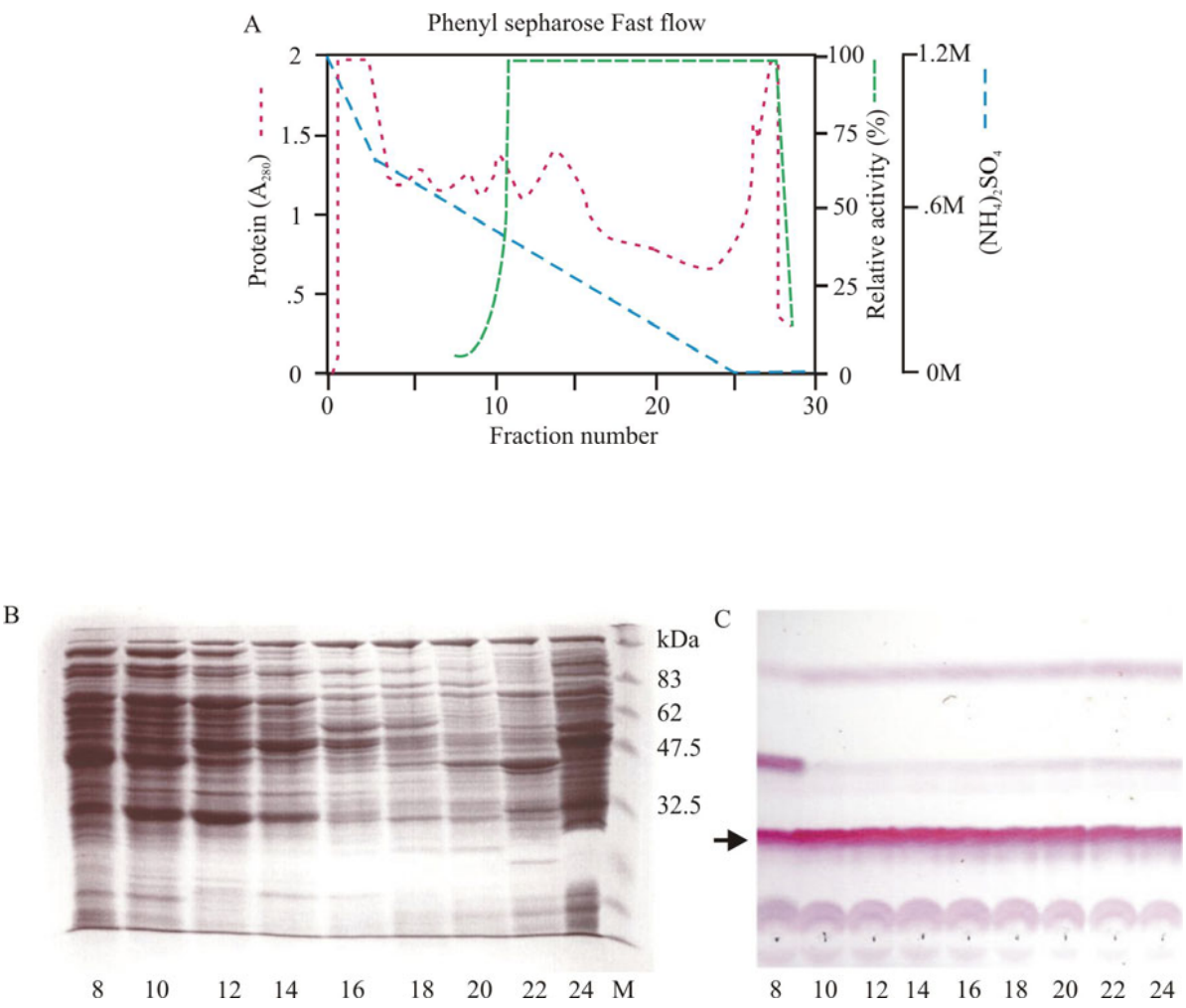


Figure 3.27 Hydrophobic interaction chromatography Phenyl sepharose

A suspension of proteins (not binding to Ni-NTA, see Materials and methods) was adjusted the salt concentration to 1.2 M $(NH_4)_2SO_4$ and it was applied to the HIC chromatography, Phenyl sepharose Fast flow HR 10/10 (**A**). The bound proteins (red dashed line) were eluted with a descending gradient from 60-0% $(NH_4)_2SO_4$ (blue dashed line), 10 column volumes (CV) in 25 mM Tris- HCl pH 7.5. Aliquots of eluted fractions were tested for the fusidic acid-converting activity (green dashed line), and the assays were analyzed by TLC The assay was done in 50 mM Na-phosphate pH 7, 5 µg fusidic acid, 2 h/ 37°C.

B SDS-PAGE M-8S protein marker, the numbers from 8 to 24 represent the fractions (volume 4ml).

C- TLC analysis of the fusidic acid conversion, using isolated HIC fractions. The arrow indicates a product of conversion with Rf 0.24.

Fractions No 16 to 20 (Fig. 3.27B) from the HIC Phenyl sepharose (Fig. 3.27A) were rechromatographed on HIC Phenyl resource (Fig. 3.28 A).

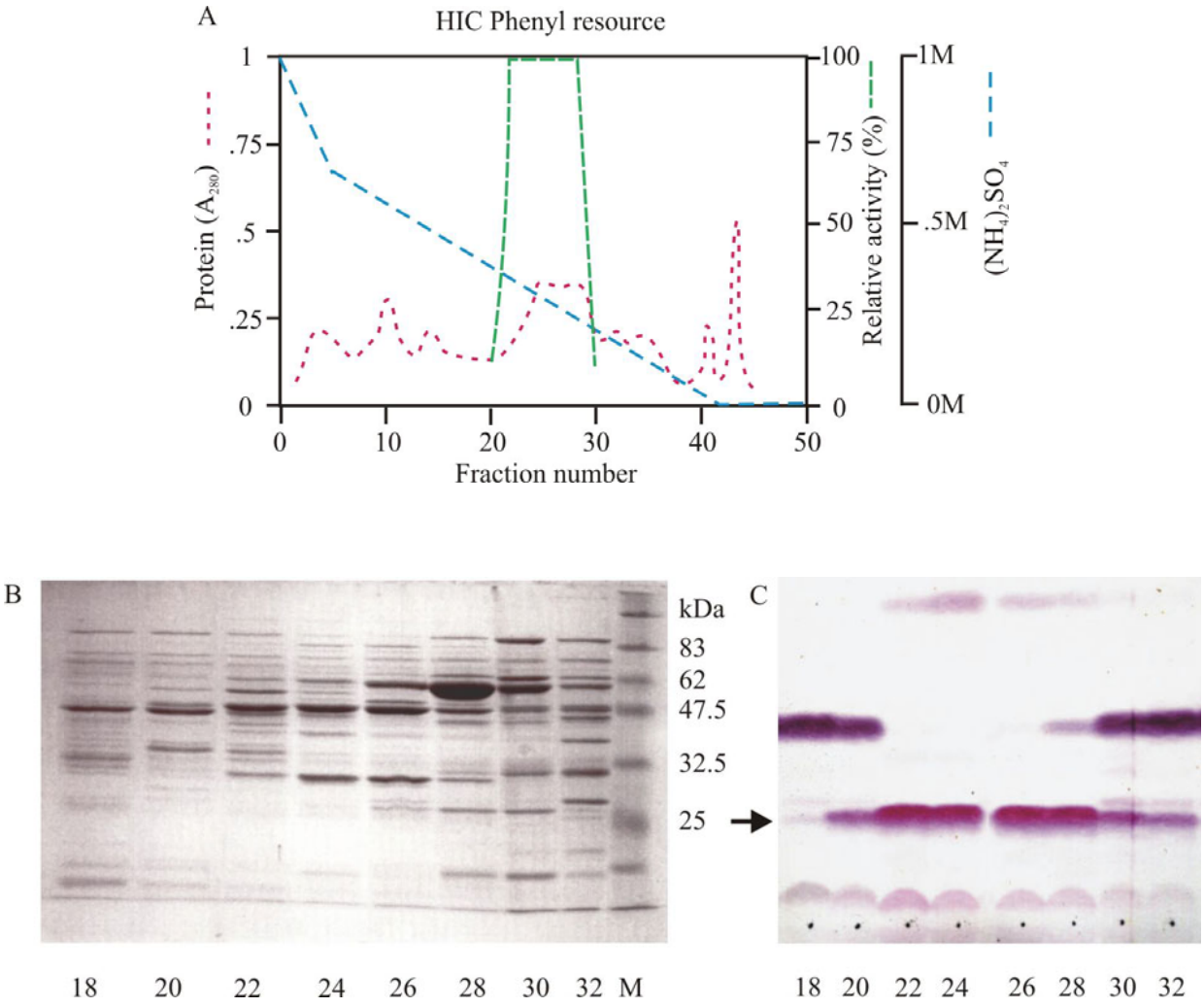


Figure 3.28 Hydrophobic interaction chromatography Phenyl resource

Fractions 16 to 20 from the previous purification (Fig. 3.27A) were collected. The salt $(NH_4)_2SO_4$ concentration of the sample was adjusted to 1.0 M and the sample was directly applied to HIC Phenyl resource (HR5/5) (**A**). The bound proteins (red dashed line) were eluted with a descending gradient from 60-0% $(NH_4)_2SO_4$, 16 column volumes (CV) in 25 mM Tris- HCl pH 7.5 (blue dashed line). Aliquots of eluted fractions were tested for the fusidic acid-converting activity (green dashed line), and the assays were analyzed by TLC (see Fig. 3.27).

B- SDS-PAGE M-protein marker 8S, numbers represent the isolated fractions (volume 500 µl).

C- TLC analysis of the fusidic acid-conversion. Fractions from the HIC Phenyl resource were tested on the fusidic acid-conversion and analyzed by TLC. The arrow shows obtained product Rf 0.24.

Active fractions (21 to 28) from the HIC Phenyl resource were collected into 3 pools (A-21 to 23; B-24 to 26 and C-27 to 28) according to the SDS-PAGE and TLC analysis and further separated by Anion exchange chromatography (Fig. 3.29).

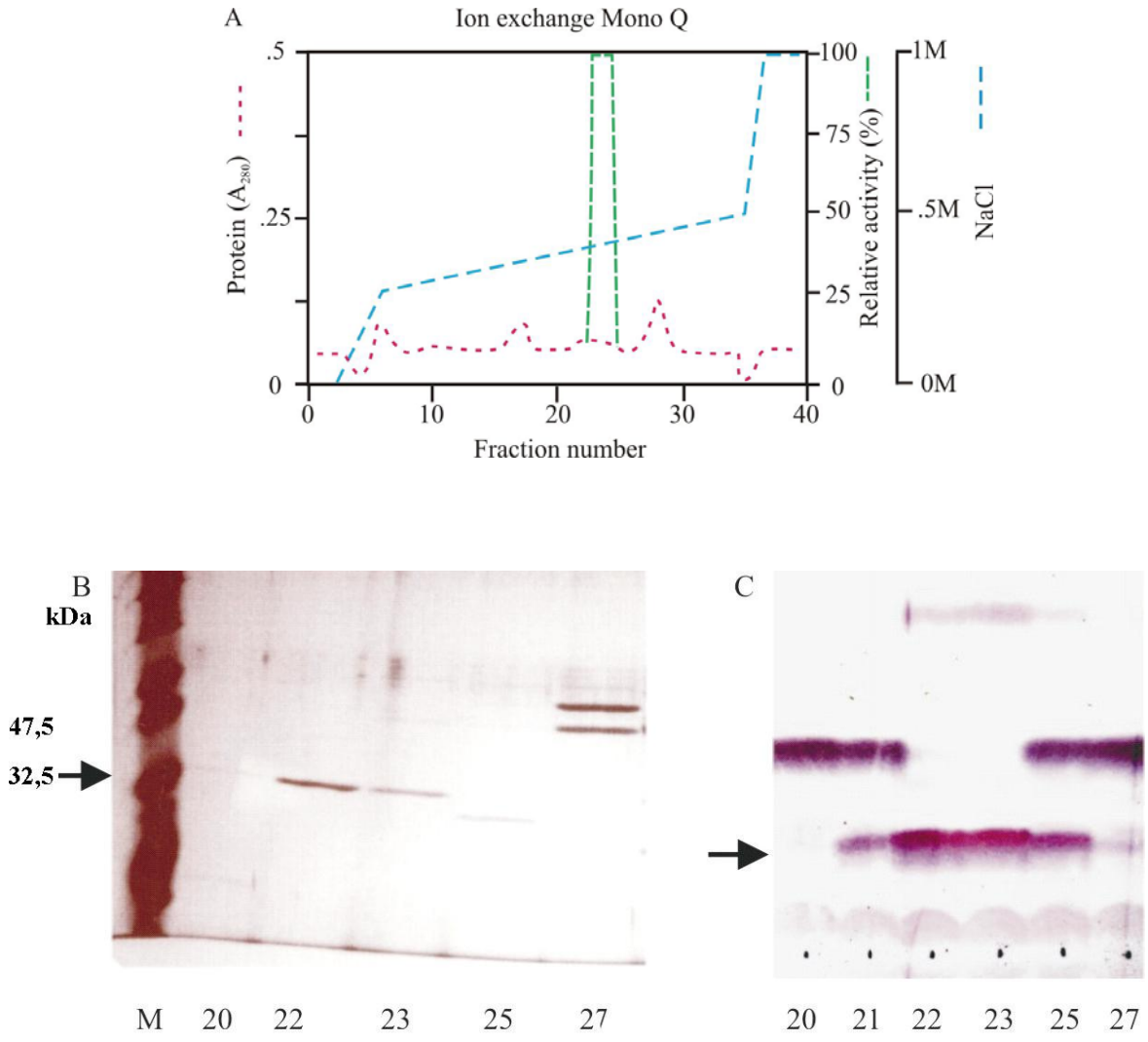


Figure 3.29 Anion exchange Mono Q chromatography

Pool A containing fractions 21 to 23 from HIC Phenyl resource (see Fig. 3.28B) were diluted 10x with starting buffer (25 mM Tris- HCl pH 8) and applied on anion exchange MonoQ chromatography pH 8 (**A**). With the ascending linear gradient (20-45%, 18 CV in 25 mM Tris- HCl pH 8, fraction volume 500 µl, blue dashed line), the proteins (red dashed line) were successfully separated, analysed by SDS-PAGE (**B**) and for the fusidic acid-converting activity (**C**, green dashed line). Active fractions (22 and 23) were eluted from the MonoQ column with the 0.36 M NaCl.

B- SDS-PAGE (silver staining), M-protein marker 8S, the numbers represent the isolated fractions (fraction volume 500 µl). The arrow assigned a single protein band (~32 kDa) in the active fractions 22 and 23.

C- Conversion of the fusidic acid. Isolated fractions were inspected for the ability to convert fusidic acid. The enzyme assays were done as described in Fig. 3.28 and analyzed by TLC. The arrow shows the product Rf 0.24 obtained from the fusidic acid-conversion.

5.2 Characterization of the enzyme FusK

A small amount of the pure protein (size about 32 kDa) that converts the fusidic acid was obtained. It was tested for cofactor dependence, temperature- and pH- optima (Fig. 3.30).

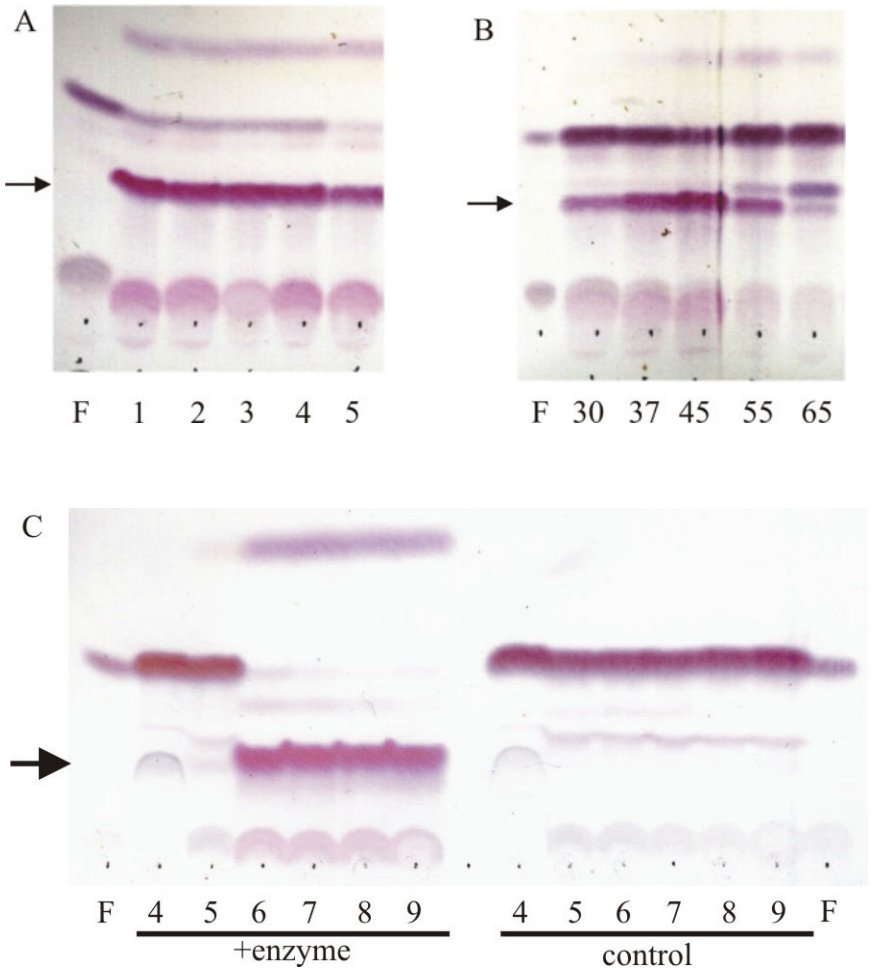


Figure 3.30 Analysis of the enzymatic activity of the 32 kDa FusK protein

A- Test of the 32 kDa FusK protein for the fusidic acid-conversion in the presence of different cofactors. Tested cofactors used in final concentration 1 mM were the following: 1.NAD; 2.NADPH; 3.NADH; 4.FAD 5-reaction mix without cofactor. Reaction assay was 50 mM Na-phosphate pH 7, 5 µg fusidic acid 4 h/ 37^{o}C. The enzyme assays were analyzed by TLC. F- fusidic acid,. The arrow shows obtained product Rf 0.24.

B- Conversion of the fusidic acid at different temperatures by FusK. Reaction assay was done 2.5 h at indicated temperatures (30, 37, 45, 55, 65^{o}C), in 50 mM Na-phosphate pH 7, containing mix of 1 mM cofactors mentioned in figure A. F- fusidic acid, the numbers represent the tested temperatures. The arrow shows the expected product Rf 0.24.

C- Conversion of the fusidic acid at different pH by FusK. Reaction assay was done 4 h/ 37^{o}C at indicated pH 4 (25 mM Na-succinate), pH 5 (25 mM Na-citrate), pH 6, pH 7 and pH 8 (50 mM Na-phosphate) and pH 9 (50 mM Tris-HCl). Control reaction was done in the same reaction buffer under the same conditions, but enzyme was omited. F- fusidic acid, the numbers represent the tested pH. Arrow shows the expected product Rf 0.24.

As shown (Fig. 3.30), FusK converts fusidic acid and the catalysis is independent of the used cofactors. The highest conversion was between 37 and 45°C. At 65°C another substance appears independent of the presence (B) or absence (data not shown) of the protein. This is due to the non-enzymatic conversion in the presence of the cofactors. Optimal pH for the conversion of fusidic acid is from pH 6 to pH 9. Immunological studies revealed that FusK is not related to the previously characterized enzyme esterase, FusH (von Haar et al. 1997) (Fig 3.31).

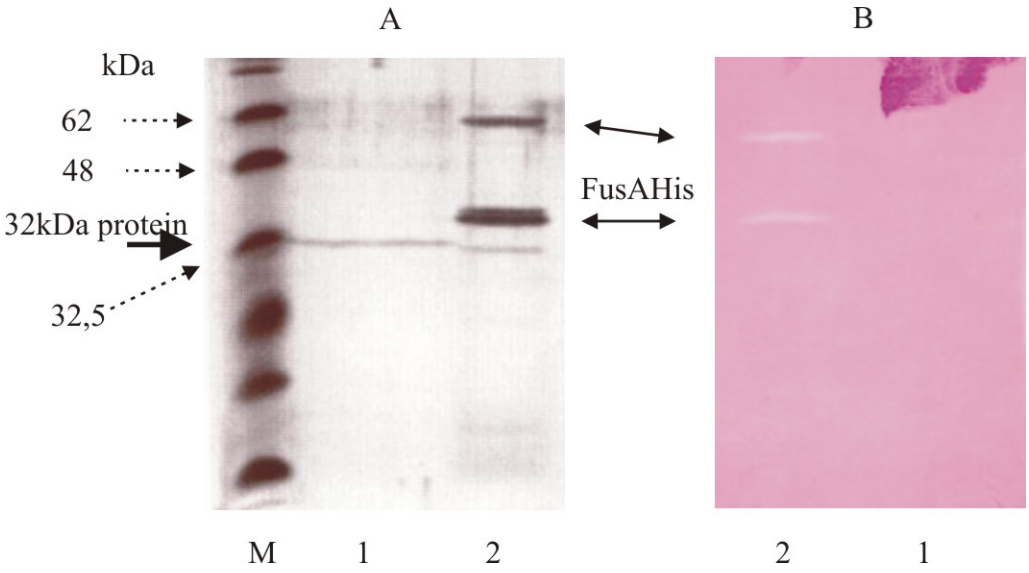


Figure 3.31 Immunodetection of FusA* and FusK, using *anti*-FusH antibodies

A- SDS–PAGE M- protein marker 8S, 1-32 kDa FusK, 2-FusA*, **B**-Western blot using the *anti*-FusH antibodies

6. Cloning and characterization of the *fus*G gene, coding for a putative hydroxyacyl-CoA dehydrogenase

The enzyme FusG was shown to be involved in the biotransformation of the lactone derivative. In order to further study this enzyme at a molecular level, it was necessary to clone and characterize its encoding gene. The purified protein was found to have the following NH_2 terminal sequence (Dr. H. Hippe from company Chromatech):

TGIXGDIARVGVVGXGQMGAGIAEVXARSGLEVM

X- represents undetermined amino acids in the sequence.

Search for the similarity (Blast search) through the *S. coelicolor* A3(2) Genome Sequence Database (Bentley et al. 2002), revealed that there is a high identity of the NH_2-terminal sequence of FusG with the deduced NH_2-terminal sequence of the putative 3-hydroxyacyl-CoA dehydrogenase (gene SCI35.13; 91% identity; cosmid I35; length 303 aa) and to a lesser

extent with another putative 3-hydroxyacyl-CoA (gene SCBAC19F3.11; identity 65%; cosmid BAC19F3, length 289 aa) (Fig. 3.32).

```
                        *         20         *
FusG          :  TGIXGDIARVGVVGXGQMGAGIAEVXARSGLEVM-- : 34
SCI35.13      :  TGISGDIARVGVVGCGQMGAGIAEVCARSGLEVM-- : 34
SCBAC19F3.    :  --MSGGIRRVGVVGGGRMGAGIAEVCARAGLDTV-- : 32
                 tgisGdIaRVGVVG GqMGAGIAEVcARsGLevm
```

Figure 3.32 The homologues of FusG from the Genome Database of *S. coelicolor* A3(2)

The deduced protein homologues coded by genes SCI35.13 and SCBAC19F3 were acquired from the search of Genome Database of *S. coelicolor* A3(2). The search was based on the NH_2-terminal protein sequence of FusG. The protein sequences were aligned so that the identical residues present in all three sequences are black and similar ones are gray shadowed. The consensus sequence is signed below the alignment (capital letter is identical residue, small letter similar one). The X represent undefined residue in the NH_2-terminal protein sequence of FusG, (-) is the gap in alignment**.**

6.1 Cloning of the *fusG* gene from the *S. lividans*

The cloning was done by creating the primer pair: forward primer FG1 and reverse primer FG2 (see page 13, Oligonucleotides), according to the NH_2-terminal sequence of purified FusG and the end of the gene SCI35.13 from *S. coelicolor* A3(2). As a template DNA served the chromosomal DNA from the *S. lividans* strain. Conditions for amplifying the *fusG* gene were selected according to the characteristics of the primer pair (Fig. 3.33).

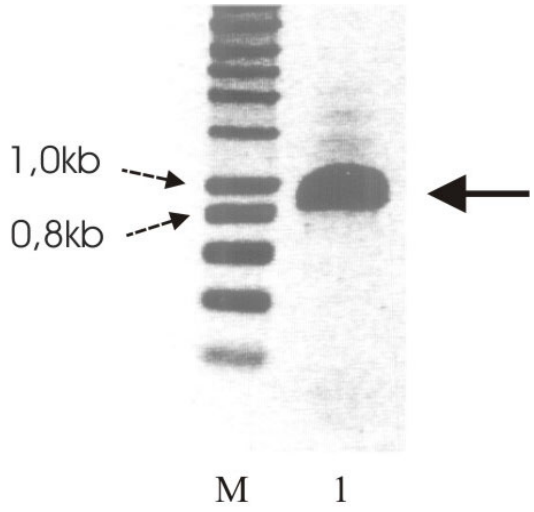


Figure 3.33 Amplification of the *fus*G gene

Touchdown PCR conditions were the following: hot start, initial denaturation 96°C/ 3 min., denaturation 96°C/ 30 sec, annealing 75-65°C/ 45 sec, polymerization 72°C/ 90 sec, 29 cycles using *Taq* polymerase, template: chromosomal DNA of *S. lividans.* The desired fragment corresponding to the size of *fus*G is marked by a full arrow. M-DNA marker "Smart ladder" (Eurogentec).

A PCR fragment, about 900 bp in length was obtained. After purification the DNA fragment was directly ligated with vector pDrive. Subsequently ampicillin resistant *E. coli* transformants were selected.

6.2 Sequencing the *fus*G gene from pDFG1 and its sequence analysis

Double digestion of the plasmid DNA of several transformants, with enzymes *Hind*III/*Pst*I gave a single 873 bp fragment corresponding to the cloned gene *fus*G. According to the restriction analyses, the plasmid DNA from 4 different transformants was sequenced. The sequence of the *fus*G gene from the transformant pDFG1 showed 100% identity with the sequence of the gene SCI35.13 (*fus*G, *S. coelicolor* A3(2) Genome sequence database) starting from the codon ACC coding for threonine (Table 3).

Threonine is 14 amino acids downstream from the putative start codon ATG for the gene SCI35.13 (*S. coelicolor*A3(2)). Codon usage analysis of gene SCI35.13 showed that codon GTG coding for valin could be a starting codon for the gene *fus*G (Fig. 3.34).

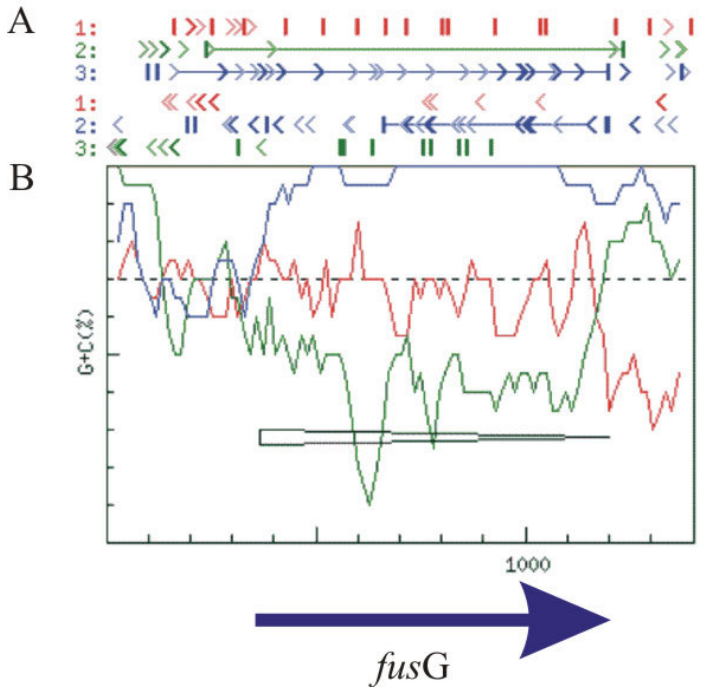


Figure 3.34 Codon Usage Analysis of the DNA sequence from *S. coelicolor* A3(2) containing gene SCI35.13

Analysis was done by the program FramePlot 3.0beta (Ishikawa and Hotta 1999). Target sequence was 1400 bp. **A-** Possible open reading frames in both directions (1, 2 and 3). The orf coding for the gene SCI35.13 is shown by 3 (blue).

B-The G+C (%) graph. The curves indicate the G+C content. (>) means the starts codons ATG or GTG, (I) are the stop codons. Numbers 1, 2 and 3 are the open reading frames in + or – direction.

Analysis also revealed a likely Shine-Dalgarno sequence, 7 bp upstream from the putative start codon GTG (Fig. 3.35).

CAGCATGCCCGACCCGGGAGACGGAACCGTGACCATTCGGGGGTAACGTGCTCG
ATCGGAGCAGGCACCGGGGACCCCGGCATGCCTGCGACCAGCCGTGGAGTCAGC
G**AAAGG**GACGATGTG

*fus*G-ATG 5`- ACCGGGGACCCCGGCATG-3'
16SrRNA 3`- UCUUUCCUCCAC -5`
*fus*G-GTG 5`-AGCGAAAGGGACGATGTG-3`

Figure 3.35 Sequence analysis of the 120 bp upstream from the start codon of FusG
Bold underlined is probable Shine-Dalgarno sequence. The potential -10 and -35 sequencies are indicated in red colour and putative starting codon ATG in green. The upstream sequences from GTG (blue) and ATG (green) are compared with the 3'-end of 16S rRNA.

The putative start codon ATG, of the gene *fus*G (*S. coelicolor*A3(2)), which is 42 bp upstream from the threonine codon is most likely not a start codon. Sequence analysis of the upstream region did not show any potential Shine-Dalgarno sequence.

Table 3 Characteristics of the *fus*G gene and its nucleotide sequence
Corresponding amino acid sequence is in three letter code written above.

Gene size *fus*G	873 bp
Protein FusG	290 aa
GC% content	68.8%
3rd-Letter GC:	95.9%

```
  1  gtgaccggca tctcgggaga tatcgcacgt gtcggcgtgg tcggctgcgg tcagatgggg
     cactggccgt agagccctct atagcgtgca cagccgcacc agccgacgcc agtctacccc
     ValThrGly IleSerGly AspIleAlaArg ValGlyVal ValGlyCys GlyGlnMetGly

 61  gcgggcatcg ccgaggtgtg cgcccgctcg ggtctggagg tgatggtcgc cgagaccacc
     cgcccgtagc ggctccacac gcgggcgagc ccagacctcc actaccagcg gctctggtgg
     AlaGlyIle AlaGluVal CysAlaArgSer GlyLeuGlu ValMetVal AlaGluThrThr

121  ggcgaggctc tggagatcgg ccgcacccgg ctgtacaact cgctggccaa ggcggccgag
     ccgctccgag acctctagcc ggcgtgggcc gacatgttga gcgaccggtt ccgccggctc
     GlyGluAla LeuGluIle GlyArgThrArg LeuTyrAsn SerLeuAla LysAlaAlaGlu

181  cgcggcaaga tgaccgagga ggagcgggac gcgacgcagg cgcgcctcag cttcaccacc
     gcgccgttct actggctcct cctcgccctg cgctgcgtcc gcgcggagtc gaagtggtgg
     ArgGlyLys MetThrGlu GluGluArgAsp AlaThrGln AlaArgLeu SerPheThrThr

241  gacctcggcg agttcgccga ccgcgatctg gtgatcgagg ccgtcgtcga gaacgagcag
     ctggagccgc tcaagcggct ggcgctagac cactagctcc ggcagcagct cttgctcgtc
     AspLeuGly GluPheAla AspArgAspLeu ValIleGlu AlaValVal GluAsnGluGln

301  gtcaagaccg agatcttcca ggtgctcgat caggtcgtga cccggccgga cgcgatcctg
     cagttctggc tctagaaggt ccacgagcta gtccagcact gggccggcct gcgctaggac
     ValLysThr GluIlePhe GlnValLeuAsp GlnValVal ThrArgPro AspAlaIleLeu

361  gcctccaaca cctcctccat cccgctggtg aagctggcgg tcgccacctc gcggcccgac
     cggaggttgt ggaggaggta gggcgaccac ttcgaccgcc agcggtggag cgccgggctg
     AlaSerAsn ThrSerSer IleProLeuVal LysLeuAla ValAlaThr SerArgProAsp

421  cacgtcatcg gcatccactt cttcaacccg gccccggtgc agcagctcgt cgagctgatc
     gtgcagtagc cgtaggtgaa gaagttgggc cggggccacg tcgtcgagca gctcgactag
     HisValIle GlyIleHis PhePheAsnPro AlaProVal GlnGlnLeu ValGluLeuIle

481  ccggcgctga ccacctccga gggcacgctc agccgggccc agctgttcac cgagaaggtg
     ggccgcgact ggtggaggct cccgtgcgag tcggcccggg tcgacaagtg gctcttccac
     ProAlaLeu ThrThrSer GluGlyThrLeu SerArgAla GlnLeuPhe ThrGluLysVal

541  ctcggcaagc acgcgatccg cgcccaggac cgctccggct tcgtggtcaa cgcgctgctg
     gagccgttcg tgcgctaggc gcgggtcctg gcgaggccga agcaccagtt gcgcgacgac
     LeuGlyLys HisAlaIle ArgAlaGlnAsp ArgSerGly PheValVal AsnAlaLeuLeu

601  atcccgtacc tgctctccgc gatccggatg ttcgagtcgg gcatcgccag ccgcgaggac
     tagggcatgg acgagaggcg ctaggcctac aagctcagcc cgtagcggtc ggcgctcctg
     IleProTyr LeuLeuSer AlaIleArgMet PheGluSer GlyIleAla SerArgGluAsp

661  atcgacaacg gcatggagat gggctgcgcc cacccgatgg gcccgctgaa gctggccgac
     tagctgttgc cgtacctcta cccgacgcgg gtgggctacc cgggcgactt cgaccggctg
     IleAspAsn GlyMetGlu MetGlyCysAla HisProMet GlyProLeu LysLeuAlaAsp

721  ctgatcggcc tggacacggt cgcctcggtg gcgtactcga tgtacgagga gtacaaggag
     gactagccgg acctgtgcca gcggagccac cgcatgagct acatgctcct catgttcctc
     LeuIleGly LeuAspThr ValAlaSerVal AlaTyrSer MetTyrGlu GluTyrLysGlu

781  ccgctgtacg ccgctccccc gctgctccag cgcatggtcg acgcgggccg cctcggccgc
     ggcgacatgc ggcgaggggg cgacgaggtc gcgtaccagc tgcgcccggc ggagccggcg
     ProLeuTyr AlaAlaPro ProLeuLeuGln ArgMetVal AspAlaGly ArgLeuGlyArg

841  aagagcggct ctggcttcta cgcctacggc tga
     ttctcgccga gaccgaagat gcggatgccg act
     LysSerGly SerGlyPhe TyrAlaTyrGly ---
```

Using the program for the peptide leader sequence analysis SignalP V1.1 (Nielsen et al. 1997), 14 amino acids (**mpatsrgvserddv**) upstream from the threonin and additionally 18 amino acids (**rdhsgvtxsigagtgdpg)** from the putative starting amino acid methionin was analysed. The program prediction comprised both Gram+ and Gram– bacteria. Results for both peptides excluded them as potential candidates for the peptide leader sequences (Fig. 3.36).

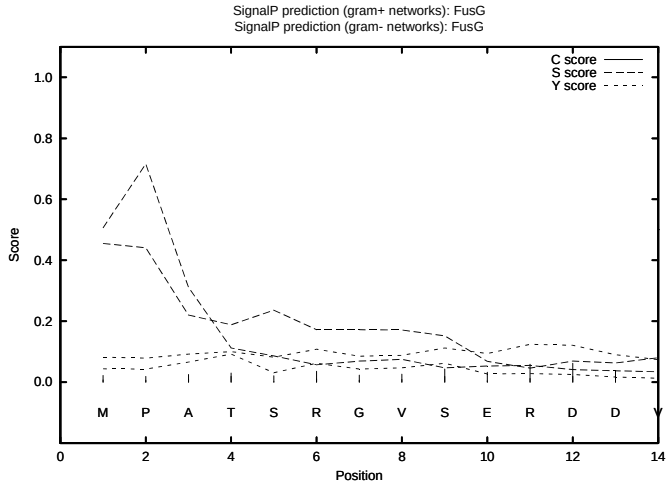


Figure 3.36 Prediction plot for the peptide leader sequence

Protein sequence **mpatsrgvserddv** between threonin and putative starting amino acid methionin was analyzed. Prediction analysis included Gram +/- bacteria. Similar plot was obtained for the protein sequence **rdhsgvtxsigagtgdpg** upstream from the putative starting amino acid methionin. Prediction analysis was done by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP).

The gene *fus*G codes for a protein of 289 amino acids, with a molecular weight of 31.162 kDa and a theoretical isoelectric point pI 5.01. The corresponding protein sequence was analysed for conserved domains against NCBI Databank and Pfam Database. The search revealed two conserved domains: a putative NAD binding domain, and a C-terminal domain of the indicative 3-hydroxyacyl-CoA dehydrogenase family (3-HDCH) (Fig. 3.37B and C). The most conserved aminoacid sequence in the NAD binding domain lies between the amino acid residues 15 and 25 (according to FusG annotation), containing a characteristic cofactor binding motif **GxxxGxG**. The most divergent domain is the C-terminal domain, which in most hydroxyacyl-CoA dehydrogenases is involved in binding of a substrate. FusG also shows a significant similarity to the FadB protein (*E. coli*) (Fig. 3.37A).

A

```
FusG    :7 DIARVGVVGCGQMGAGIAEVCARSGLEVMVAETTGEALEIGRTRLYNSLAKAAERGKMTE 66
FadB    :2 EIKKVAVIGAGVMGAGIAAVFALAGYDVVLKDISPEALERALAYIEKNLEKLVEKGKLTE 61

FusG   :67 EERDATQARLSFTTDLGEFADRDLVIEAVVENEQVKTEIFQVLDQVVTRPDAILASNTSS 126
FadB   :62 EEADAALARITPTTDLAALKDADLVIEAVVEDLELKKQVFAELEALA-KPDAILASNTSS 120

FusG  :127 IPLVKLAVATSRPDHVIGIHFFNPAPVQQLVELIPALTTSEGTLSRAQLFTEKVLGKHAI 186
FadB  :121 LSITELAEALKRPERFIGLHFFNPVPLMPLVEVIRGEKTSDETVERVVEFAKK-IGKTPV 179

FusG  :187 RAQDRSGFVVNALLIPYLLSAIRMFESGIASREDIDNGMEMGCAHPMGPLKLADLIGLDT 246
FadB  :180 VVKDVPGFIVNRLLAALLNEAIRLLEEGVATPEEIDAAMRQGLGLPMGPFELADLIGLDV 239

FusG  :247 VASVAYSMYEEYK-EPLYAAPPLLQRMVDAGRLGRKSGSGFYAY 289
FadB  :240 MLHIMKVLNETLGDDPYYRPPPLLRKLVEAGRLGRKSGKGFYDY 283
```

B

```
FusG     :7 DIARVGVVGCGQMGAGIAEVCARSGLEVMVAETTGEALEIGRTRLYNSLAKAAERGKMTE 66
3HDCH-N  :3 PVKQVAVLGAGLMGSGIAQVFASKGFPVVLVDINPKALDRALKGIAKSLKKLAKKGKLTG 62

FusG    :67 EERDATQARLSFTTDLGEFADRDLVIEAVVENEQVKTEIFQVLDQVVTRPDAILASNTSS 126
3HDCH-N :63 AEVAEVLSRISSSTNYEAVEDVDLVIEAVVENMDLKKKVFAELDAIC-KPDTILASNTSS 121

FusG   :127 IPLVKLAVATSRPDHVIGIHFFNPAPVQQLVELIPALTTSEGTLSRAQLFTEKVLGKHAI 186
3HDCH-N:122 LPISEIASATKRPERVIGMHFFNPVPVMKLVEVIRGEKTSPETIATVVALSKKI-GKTPV 180

FusG   :187 RAQD 190
3HDCH-N:181 VVKD 184
```

C

```
FusG    :193  GFVVNALLIPYLLSAIRMFESGIASREDIDNGMEMGCAHPMGPLKLADLIGLDTVASVAY 252
3HDCH-C :  1  GFVVNRLLAPLLNEAIRLVEEGVATPEDIDAAMRLGLGLPMGPFELSDLVGLDVGLHILE  60

FusG    :253  SMYEEYKEPLYAAPPLLQRMVDAGRLGRKSGSGFYAY  289
3HDCH-C : 61  VLAAEFGDRDYAPSPLLEKLVEAGRLGRKTGKGFYKY   97
```

Figure 3.37 Conserved Domain (CD) analysis of FusG (*S. lividans*)

FusG protein sequence is aligned with the conserved domains of the FadB protein (*E. coli*), NH_2- terminal domain (**A**), NAD-binding domain (**B**) and C-terminal domain (**C**) of 3-hydroxyacyl-CoA dehydrogenase (3-HDCH). Identical amino acids in alignment are in red and the similar ones are in blue. The analysis was done by Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/), using NCBI Databank and Pfam Database (Sanger Institute, http://www.sanger.ac.uk/Software/Pfam/index.shtml).

A-conserved domain COG1250, CD-Length 307 residues; 91.9% aligned. **B**- Conserved Domain pfam 02737, NAD binding domain. CD-Length = 185 residues, 98.4% aligned. **C**-conserved Domain pfam 00725, C-terminal domain. CD-Length = 97 residues, 100% aligned

The 3-hydroxyacyl-CoA dehydrogenase (3-HDCH, EC: 1.1.1.35) is an enzyme involved in fatty acid metabolism, and it catalyzes the reduction of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA. In *E. coli* (gene *fad*B) (Fig. 3.37A) and *Pseudomonas fragi* (gene *fao*A), 3-HDCH is a part of a multifunctional enzyme which also contains an ECH/ECI domain [enoyl-CoA hydratase (ECH) and 3,2-trans-enoyl-CoA isomerase (ECI)] as well as a 3-hydroxybutyryl-CoA epimerase domain.

6.3 Cloning of the *fusG* gene in *E. coli*

For heterologous expression in *E. coli*, the gene *fus*G was cloned by PCR into the *Nde*I/*Xho*I restriction sites of the vector pET24a. For this purpose the following primers were designed: forward FG*Nde*I and reverse FG*Xho*I. (see page 13, Oligonucleotides) (Fig. 3.38).

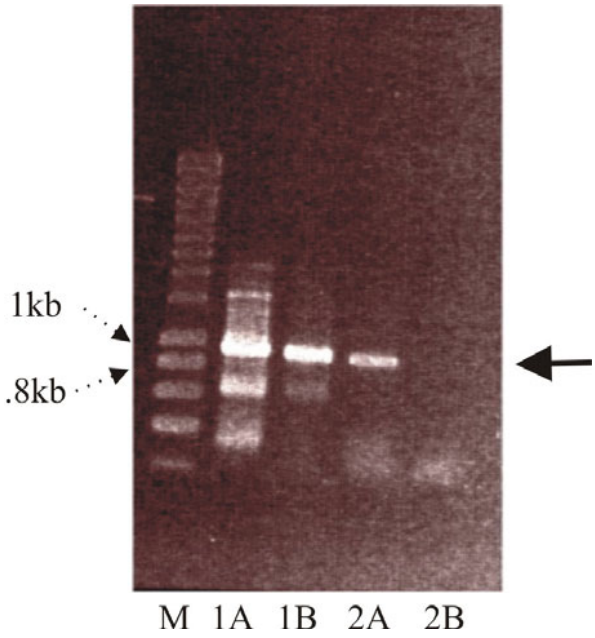


Figure 3.38 Amplification of the *fus*G gene
Touchdown PCR conditions were the following: Hot start, initial denaturation 96°C/ 3 min., cycle denaturation 96°C/ 30 sec, annealing. 75-65°C/ 45 sec, polymerization 72°C/ 90 sec, final extension 72°C/ 10 min., 29 cycles using *Taq* polymerase, template: chromosomal DNA of *S. lividans*. The solid arrow shows the amplified DNA fragment corresponding in size to the *fus*G gene. 5% DMSO +/- (lanes 1 and 2); 3.5 mM Mg^{2+} ions +/- (lanes A and B). M-marker "Smart ladder" (Eurogentec).

The expected PCR fragment (Fig. 3.38-2A), approximately 0.9 kb was obtained and after digestion with *Nde*I and *Xho*I was ligated with the *Nde*I/*Xho*I cleaved pET24a vector. *E. coli* pTFG2 transformants (kanamycin-resistant) were analysed for plasmids carrying the cloned *fus*G gene (Fig. 3.39). In order to confirm the correct PCR cloning of the *fusG* gene, plasmid DNA of several pTFG2 transformants was analysed using *Nde*I/*Xho*I double digestion and the desired ones were further sequenced using universal primers: pUC/M13 (see page 13,

Oligonucleotides). Analysis of the sequence of the *fus*G from pTFG2 confirmed successful cloning (Appendix A).

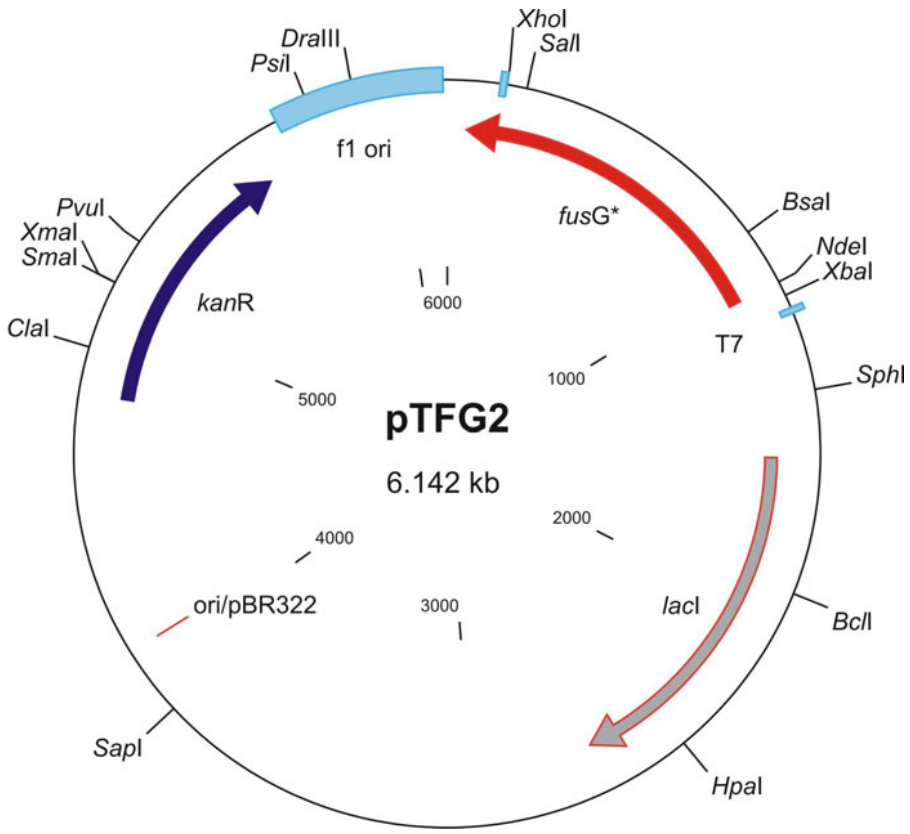


Fig 3.39 Contruction of the pTFG2

The pTFG2 was obtained by cloning the 0.9 kb PCR fragment cleaved with the *NdeI*/*Xho*I into the Nde*I*/*Xho*I cleaved vector pET24a (Novagen) (Appendix B).

6.4 Cloning of the *fus*G* gene in *S. lividans*

In order to overcome problems that might be encountered with the expression of the native FusG in *E. coli*, the *fus*G was cloned by PCR in *S. lividans* using shuttle vector pWHM3 (Appendix C). To simplify the later purification of FusG* (FusG containing 6 histidine residues at the C-terminus), 6 histidine codons were additionally cloned at the 3' end of the gene (named *fus*G*). The forward primer "FGpWHM3f" was designed according to the sequence 250 bp upstream from the start codon for the gene *fus*G. The reverse primer "FGpWHM3r" was designed at the end of the gene containing "in frame" codons for 6 histidines and the restriction site *Xba*I (see page 13, Oligonucleotides). The second cloning restriction site *Bam*HI was 154 bp upstream of the *fus*G gene (Fig. 3.40).

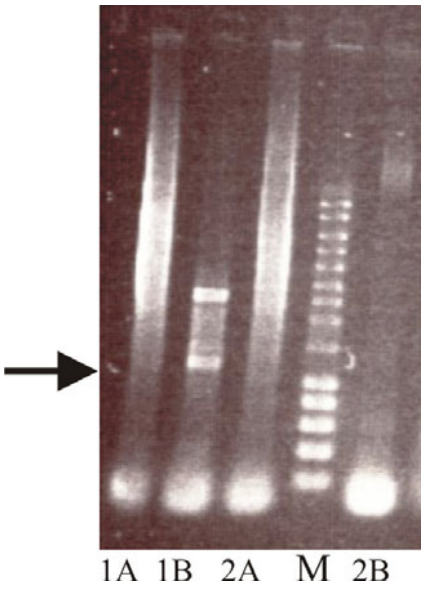


Figure 3.40 Amplification of the *fus*G* gene

Touchdown PCR conditions were the following: Hot start, initial denaturation 96°C/ 3 min., cycle denaturation 96°C/ 30 sec, annealing. 75-65°C/ 45 sec, polymerization 72°C/ 180 sec, final extension 72°C/ 5 min., 25 cycles using *Pfu*I polymerase, template: chromosomal DNA of *S. lividans*. 5% DMSO +/- (1 and 2), 3.5 mM $MgSO_4$ +/- (A and B). M-marker "Smart ladder" (Eurogentec). Among several obtained PCR fragments (thick arrow) the desired one about 1.2 kb that corresponds in size to *fus*G* (the gene *fus*G containing 6 histidine codons at 3' end) was purified for further cloning.

The DNA fagment (sample 1B) in the size of approximately 1.2 kb was purified, digested with *Bam*HI/*Xba*I and cloned in *Bam*HI/*Xba*I cleaved pWHM3 vector (Fig. 3.41). Strain *E. coli* DH5α was transformed and the plasmid DNA of obtained transformants, were analysed by the restriction enzymes *Bam*HI/*Xba*I. Having the correct restriction pattern, the plasmid DNA of pWFG1 from 2 transformants was sequenced in order to confirm the authenticity. The plasmid DNA pWFG1 was used for transformation of *S. lividans* protoplasts. Selection of the transformants was based on their resistance against the thiostrepton and their plasmid DNA was found to be correct (Appendix-D).

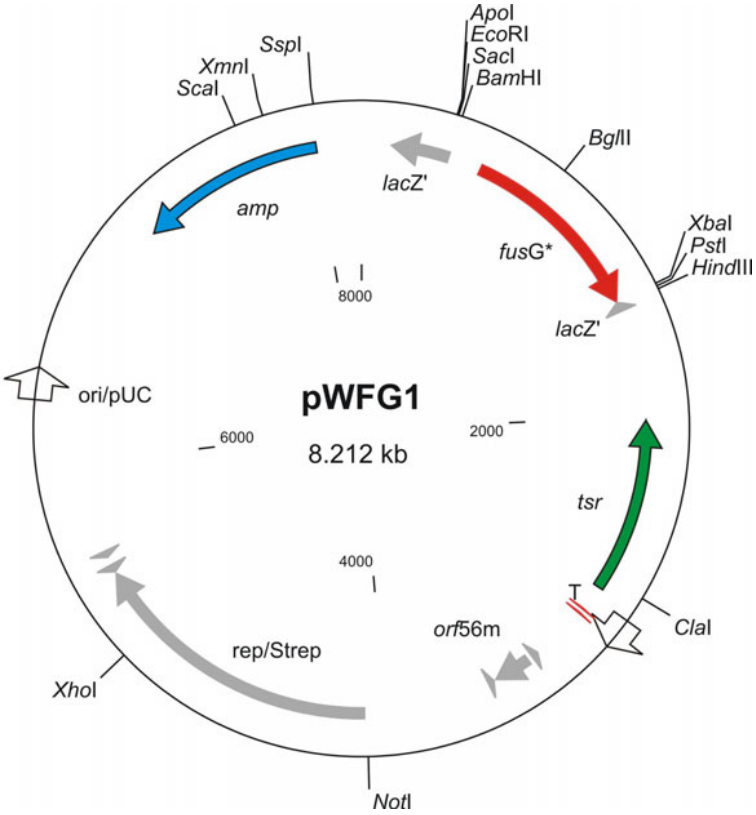


Fig 3.41 Contruction of the pWFG1

pWFG1 was obtained by cloning the 1.2 kb PCR fragment cleaved with *Bam*HI/*Xba*I into *Bam*HI/*Xba*I cleaved vector pWHM3 (Appendix C). In the figure selected restriction enzymes are shown.

7. Cloning of the *fus*A gene *from S. lividans*

As previously pointed out (Fig. 3.9), one of the purified conversion products could have arisen by the enzymatic activity of a dehydrogenase. During previous studies (Richter, Diploma Thesis 1999) it was shown, that a gene encoding a putative dehydrogenase (named FusA) was located 2 kb upstream of the *fusH* gene. In order to determine the role of this protein (FusA) in the biotransformation of fusidic acid, a PCR cloning of the gene and a further biochemical characterization of its product were a prerequisite.

7.1 Cloning of the *fus*A* gene in *S. lividans*

The *E. coli* strain with the plasmid pBSK5 that contains 6 kb chromosomal DNA of *S. lividans* served as a source of the *fus*A gene (Richter, Diploma Thesis 1999). On the basis of the known *fus*A sequence a primer pair for its cloning in the pUKS10 vector was designed and calculated (program Vector NTI Suite). The forward primer (FA*Sgf*I) has a *Sgf*I

restriction site and reverse primer (FA*Xho*I/His) contains *Xho*I restriction site and a codons for 6 histidines (see page 13, Oligonucleotides).

In studies of the catalase-peroxidase operon it was found that the *furS* gene contains a strong promoter (Ortiz et al. 2000, Zou et al. 1999). For this reason it was planned to clone the *fusA* gene under the control of this promoter. Cloning was done in 2 steps:

a) PCR cloning of the *fusA* gene containing codons for 6 histidines at the 3' end (*fus*A*) into the pUKS10 vector, downstream of the *furS* promoter, using *Sgf*I/*Xho*I restriction sites. The resulting *E. coli* transformants harboured the construct pDFA1.

b) Subcloning the the *EcoR*I/ *Hind*III DNA fragment from pDFA1 containing the *fur*S promoter in frame with the *fus*A* construct in the shuttle vector pWHM3. The generated construct was named pWFA1.

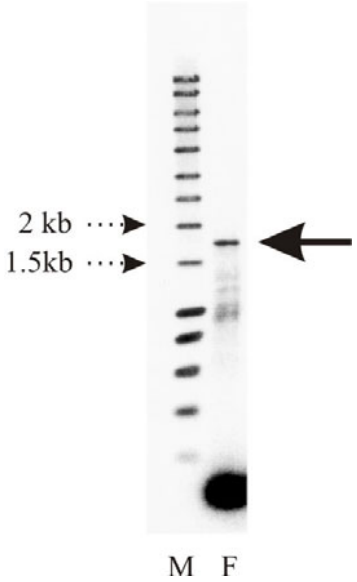


Figure 3.42 Generation of the *fus*A* gene

Touchdown PCR conditions were the following: Hot start, initial denaturation 96°C/ 3 min., denaturation 96°C/ 30 sec, annealing 60-50°C/ 45 sec, polymerization 72°C/ 90 sec, 29 cycles using *Taq* polymerase, template: the construct pBSK5.0 carrying the *fusA* gene. The main PCR product is marked by the solid arrow.

As expected a PCR fragment, approximately 1.7 kb length was obtained (Fig. 3.42). The purified DNA fragment (digested with *Sgf*I and *Xho*I) was ligated into the *Sgf*I/*Xho*I cleaved pUKS10 vector (Fig. 3.43). Subsequently ampicillin resistant *E. coli* transformants were selected.

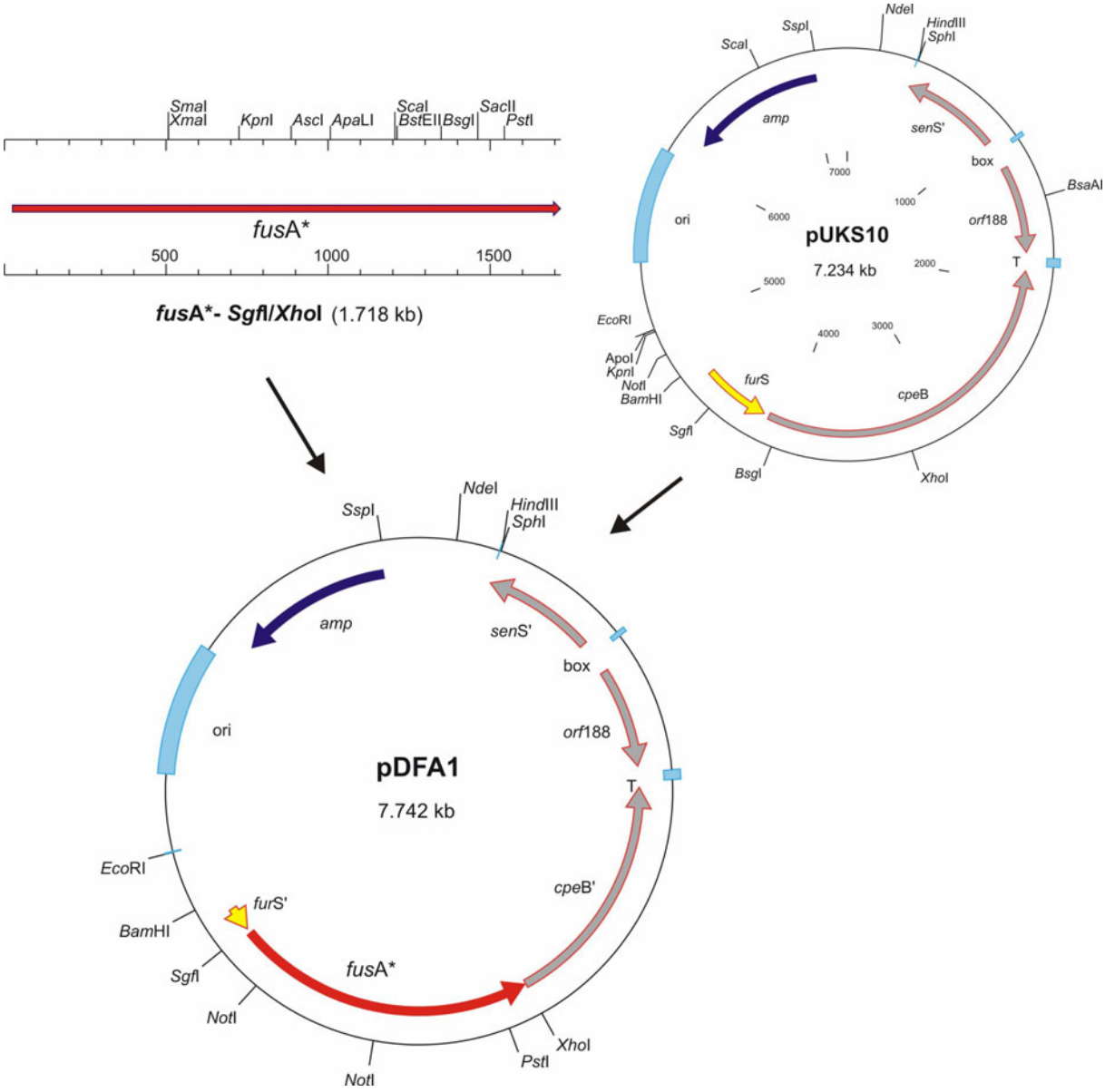


Figure 3.43 Contruction of the pDFA1

The pDFA1 containing the *fus*A* gene was obtained by cloning the 1.7 kb *Sgf*I/*Xho*I PCR fragment into the *Sgf*I/*Xho*I cleaved vector pUKS10 containing *furS* promoter. In the figure only the selected restriction enzymes are shown.

The plasmid DNA of 12 transformants was analyzed using the restriction enzyme *Not*I. Several of them were shown to have the expected constuct containing the 871 bp *Not*I DNA fragment.

Using the primers: PUDAF, PUDAR, pDa int forw1 and pDa int rev1 (see page 13, Oligonucleotides), sequencing was done by PCR in two steps (primer walking). It was shown

that the insert within the isolated plasmid DNA of 4 independent transformants was corresponding to the desired one. The construct was named pDFA1 (see Appendix E).

The *Eco*RI/*Hind*III DNA fragment of pDFA1, containing the gene *fus*A-fused with 6 histidine codons (*fus*A*) and the *furS* promoter, was ligated with the *Eco*RI/*Hind*III cleaved shuttle vector pWHM3 (Fig. 3.44). Using the restriction enzyme *Not*I the plasmid DNA of several transformants was found to correspond to the desired construct pWFA1. *S. lividans* was transformed with pWFA1 and thiostrepton-resistant transformants were selected.

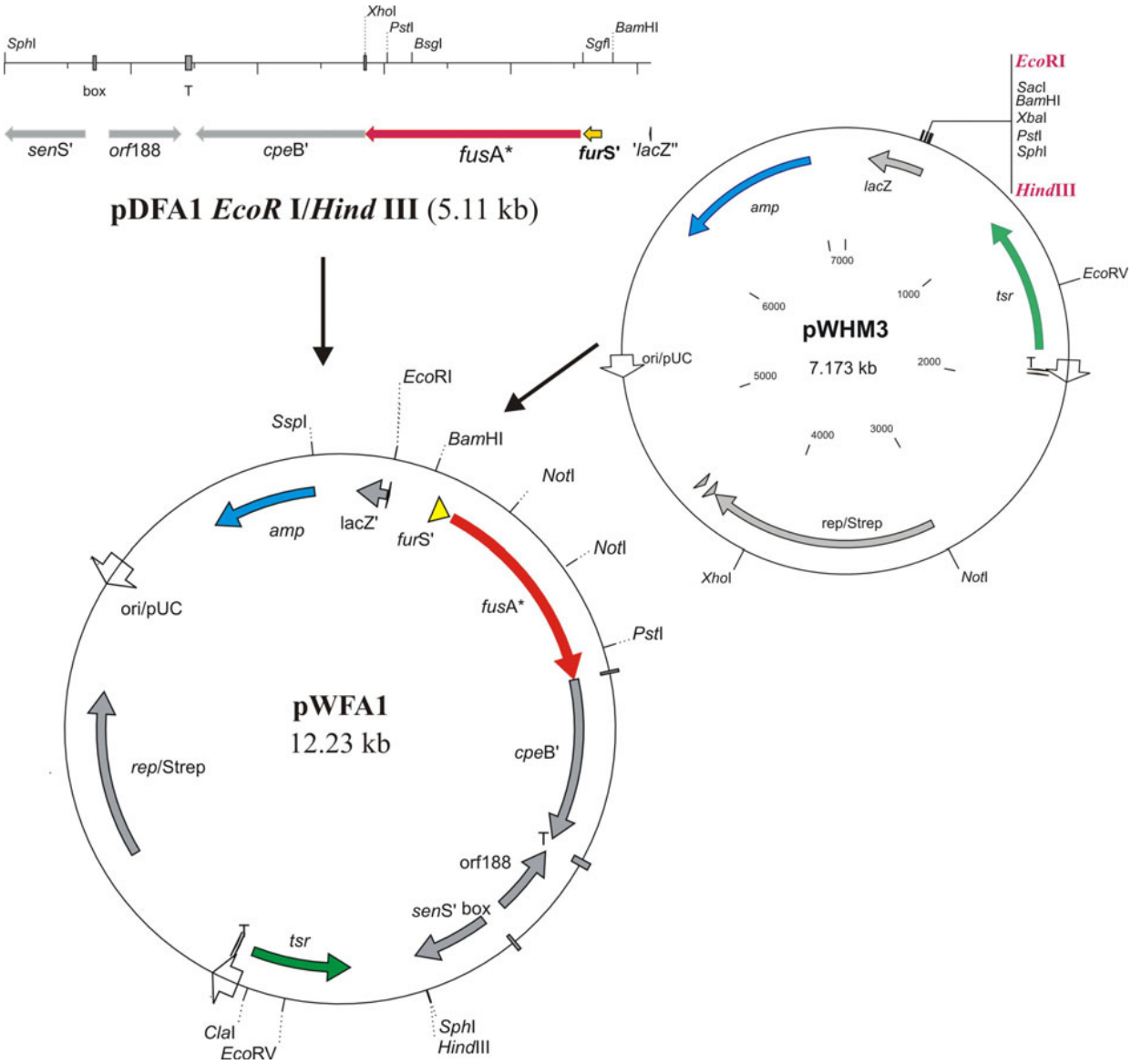


Figure 3.44 Construction of pWFA1

A 5.1 kb *Eco*RI/*Hind* III fragment containing the *fus*A* gene and the *fur*S promoter from pDFA1 was cloned into the vector pWHM3. In the figure only the selected restriction enzymes are shown.

7.2 Cloning of the *fusA* gene in *E coli*

For heterologous expression in *E. coli*, the *fus*A gene was cloned by PCR into the *Nde*I/*Xho*I restriction sites of vector pET21a (Fig. 3.45).

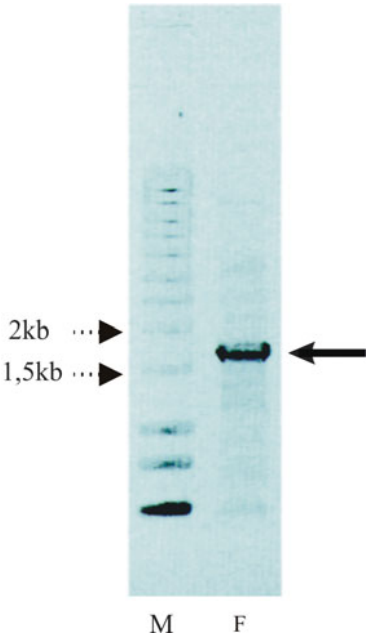


Figure 3.45 Amplification of the *fus*A gene

The touchdown PCR conditions were optimized according to the characteristics of primers FA*Nde*I and FA*Xho*I (see page 13, Oligonucleotides): initial denaturation 96°C/ 3 min., denaturation 96°C/ 30 sec, annealing 80-70°C/ 45 sec polymerization 72°C/ 3 min., 29 cycles using *Pfu*I polymerase. The plasmid DNA pBSK5 containing the *fusA* gene served as a template. The desirable amplified DNA fragment is marked by the arrow (lane F).

The expected PCR fragment (about 1.7 kb) was obtained and after cleaving with the enzymes *Nde*I/*Xho*I, the DNA was ligated into the vector pET21a (Novagen) and transformed into *E. coli.*

According to the restriction analysis, the plasmid DNA from several transformants had the correct insert. Primer walking sequencing using designed primers: pDA int forw1, pDa int rev1 (see page 13, Oligonucleotides), revealed that the plasmid DNA from four selected transformants contained the correct sequence of the *fus*A gene with 6 histidine codons at the 3' end and that it corresponded to the described one from pBSK5 (Richter, Diploma Thesis 1999) (see Appendix F).

8 Characteristics of the FusA* protein

8.1 Expression of the *fus*A* gene in *E. coli*

To express the *fus*A* gene, the expression strain *E. coli* BL21 (λDE3, pLysS) was transformed with plasmid pTFA1. Several of the amipicillin resistant transformants were cultivated and induced with 1 mM IPTG for 3 hours. Analysis of the total protein showed that *E. coli* transformants containing pTFA1 synthesized a protein of about 60 kDa, corresponding to the size of FusA protein containing 6 histidine residues at the C-terminus (named FusA*) (Fig. 3.46).

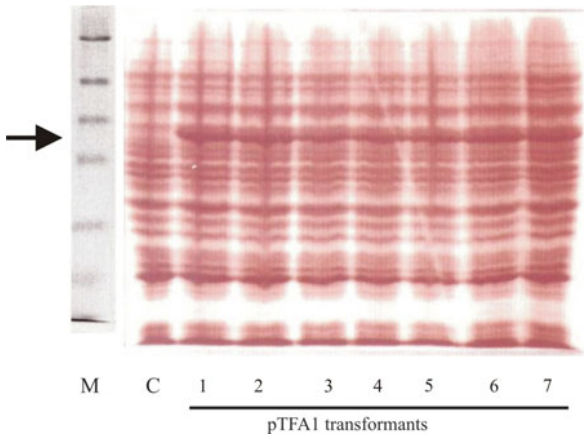


Figure 3.46 Induction of the *fus*A* gene in *E. coli*

SDS-PAGE analysis of total protein. Proteins of sonicated *E. coli* BL21 (λDE3, pLysS) transformants pTFA1, 3 hours after induction with 1 mM IPTG: **M** protein marker 8S, **C**- control strain *E. coli* with the vector pET21a, **1-7** *E. coli* transformants 1-7 with the construct pTFA1. The arrow shows the expected size (~60 kDa) of FusA*.

The *E. coli* cells may contain the overexpressed protein as inclusion bodies (insoluble) or in a soluble form. In order to enrich for the soluble fraction, the following conditions were tested: temperature of cultivation (20, 30, 37°C), time of induction (range from 30 minutes to 3.5 h), concentration of IPTG for induction (100 µM-1 mM). The analysis showed that induction with 1 mM IPTG and further cultivation for 3 to 3.5 hours at 30°C to 37°C led to the highest amount of the soluble portion of FusA* (Fig. 3.47).

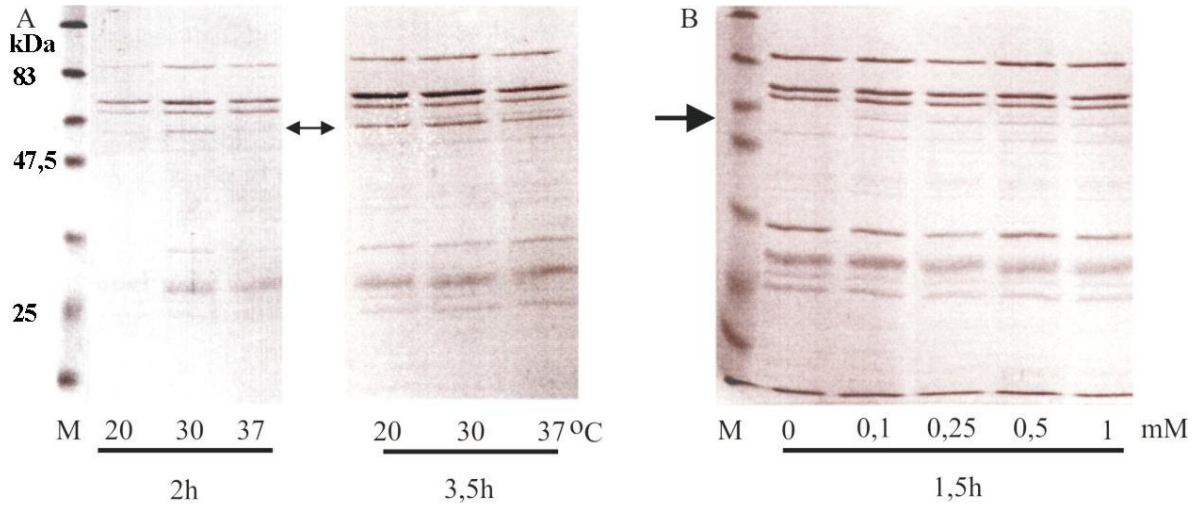


Figure 3.47 Testing the cultivation conditions for purification of FusA* protein

Affinity chromatography of protein samples from pTFA1 transformant of *E. coli* BL21. After precultivation, *E. coli* cells were either induced with 1 mM IPTG and grown for 3.5 h on different temperatures (gel **A**; 20, 30 and 37^0C), or induced with different concentration of IPTG (gel **B**; 0, 0.1, 0.25, 0.5 and 1 mM) and grown for 1.5 h on 37^0C. After sonication, centrifugation, binding (15 mM imidazole) and washing (20 mM imidazole), remaining proteins were eluted with 250 mM imidazole. The aliquots were analyzed by SDS-PAGE. M-protein marker 8S. The size of FusA* is marked by the arrows (**A, B**).

Since the enzyme FusA* contains 6 histidine residues attached at the C-terminus, a Ni-NTA affinity chromatography was used to purify the enzyme. The soluble form of FusA* is almost completely eluted from the matrix with 100 mM imidazole (Fig.3.48).

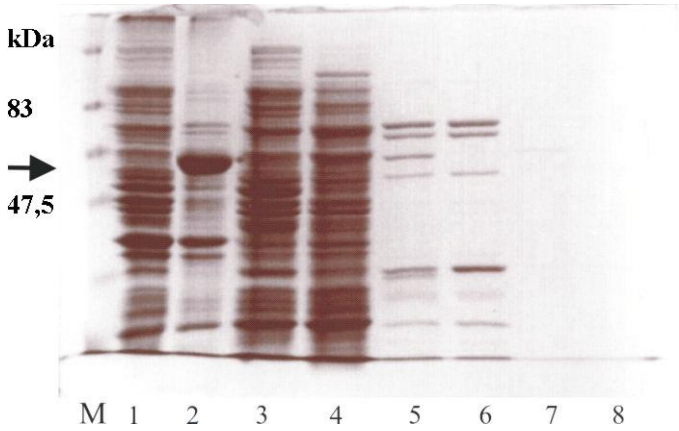


Figure 3.48 Optimization of the Ni-NTA affinity chromatography.

The *E. coli* strain carrying construct pTFA1 was propagated and induced under the standard conditions (see Materials and methods). As a control served the same strain but induction was omitted. After centrifugation, an

aliquot of the pellet of the induced (lane 2) and not induced (lane 1) transformant was treated with 1% SDS directly applied on the gel. The soluble intracellular fraction of the induced (lane 4) and uninduced (lane 3) transformant was subjected to the Ni-NTA matrix (lanes 5, 7 –induced and lanes 6, 8-uninduced). After binding (10 mM imidazole) and washing (15 mM imidazole) FusA-His tag (FusA*) was gradually eluted using 100 mM (lane 5, 6), 250 mM (lane 7, 8). Proteins were separated by SDS-PAGE: M-protein marker 8S. The arrow shows the size of FusA*.

In the presence of 250 mM imidazole two proteins were eluted. One of them has a size of FusA* protein (Fig. 3.49).

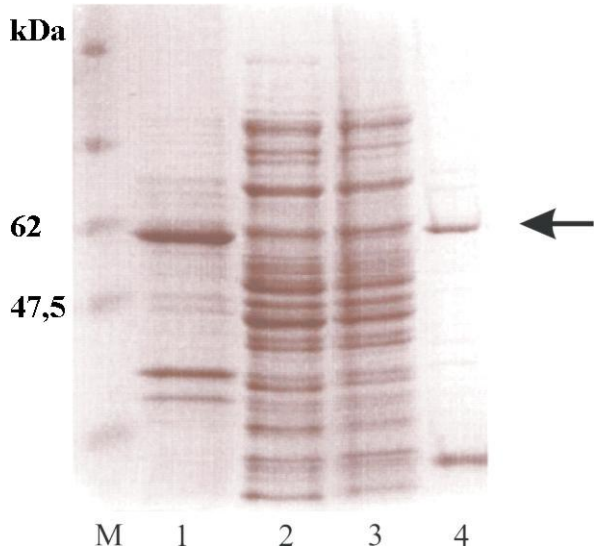


Figure 3.49 Purification of FusA-His tag (FusA*)
M- protein marker 8S, 1. pellet fraction from *E. coli* carrying pTFA1, 2. soluble unbound fraction from Ni-NTA, 3. soluble intracellular fraction, 4. eluate-I Ni-NTA 250 mM imidazole. Purified FusA* is marked by the arrow.

This fraction (Fig. 3.49, lane 4) was used to test whether the fusidic acid or lactone derivative served as a substrate. In the pH range of 5 to 8 and in the additional presence of the cofactors (FAD; NADPH; NAD and NADH) no conversion occurred. The small amount of substance B (Rf 0.35) is the result of a spontaneous reaction in the presence of cofactors (Fig. 3.50).

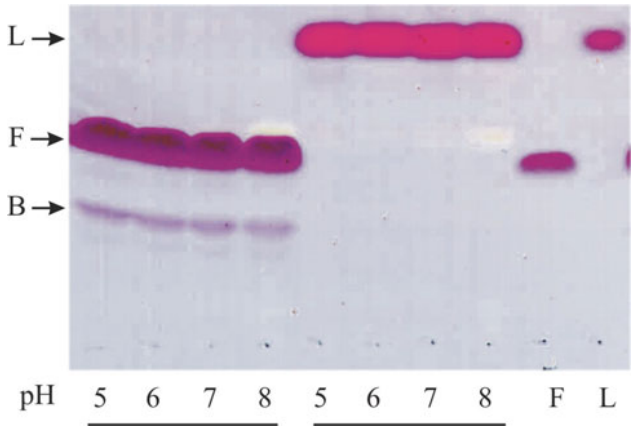


Figure 3.50 Conversion of fusidic acid and the lactone by FusA*

FusA* (Fig. 4, lane 4) was assayed in 50 mM Na phosphate pH 5-8, 37°C/ overnight in the presence of cofactors 1 mM FAD; NADPH; NAD and NADH, and with fusidic acid (F) or the lactone derivative (L). TLC analysis was done as described in Materials and methods. Substance B is a result of a non-enzymatic reaction.

8.2 Expression of the *fus*A* gene in *S. lividans*

Heterologously expressed proteins in *E. coli* could be inactive due to an improper folding. In order to test the in vivo conversion of fusidic acid, *S. lividans* pWFA1 transformants with the *fus*A* gene (gene coding for FusA having 6 histidine residues at its C-terminus) under the control of the *fur*S promoter and the control strain (strain with plasmid without the cloned *fus*A* gene) were cultivated for 35 h. In defined time intervals the aliquots of the supernatant were collected and analysed by TLC (Fig. 3.51).

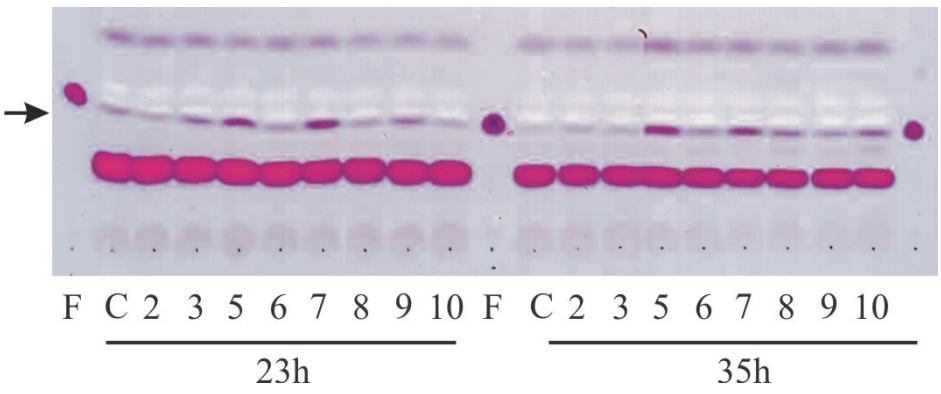


Figure 3.51 Analysis of *S. lividans* pWFA1 transformants for converting capacity for fusidic acid

Cultivation was done in the following way: 22 h germination in 10 ml 0.5% Y/MM supplemented with 5 µg/ml fusidic acid. After adding 20% fresh media (0.5% Y/MM), 5 µg/ml thiostrepton and 30 µg/ml fusidic acid

cultivation was continued by shaking for 35 h. Sampling was done after 2 h, 4 h, 6 h, 8 h, 23 h and 35 h. TLC analysis was done as described in Materials and methods. F-fusidic acid, C-control strain *S. lividans* pWHM3 and transformants pWFA1: No. 2, 3, 5, 6, 7, 8, 9 and 10. The arrow shows the new substance (Rf 0.45).

There was no significant difference in the conversion of fusidic acid among pWFA1 transformants and the control pWHM3 strain in the period of the first 8 hours after addition of fusidic acid. During prolonged cultivation (23 h and 35 h) after addition of fusidic acid, three transformants (5, 7 and 10) accumulated up to 3 to 5 times more substance that has a similar Rf value like fusidic acid (Rf 0.45) than the control strain (C). For further investigation it was necessary to purify FusA*.

8.3 Purification of FusA* from *S. lividans*

After the cultivation of the *S. lividans* transformant pWFA1 in the presence of fusidic acid, the secreted (culture filtrate), mycelia associated and intracellular proteins were analyzed for the presence of FusA* (Fig. 3.52).

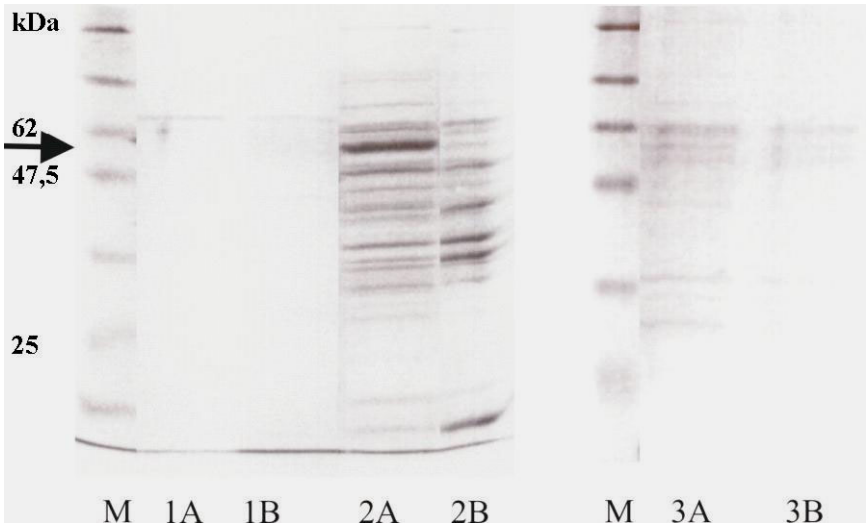


Figure 3.52 Ni-NTA purification of FusA* from different culture fractions of *S. lividans* pWFA1 (A) and pWHM3-control (B)

Proteins from the culture filtrate were obtained after $(NH_4)_2SO_4$ precipitation (90% w/v) and dialyzing against binding buffer. Mycelia associated proteins were released by washing with binding buffer containing detergent (0.1% Triton –X100). Intracellular proteins were gained from the mycelia after its sonication and removal of cell debris. All three fractions were then subjected to the Ni-NTA purification in the presence of 15 mM imidazole. The column was washed with 50 mM and then subsequently with 250 mM imidazole. Aliquots of the 250 mM fractions were analyzed by SDS-PAGE. The arrow shows the size of FusA*. M protein marker 8S, 1-supernatant fraction, 2-intracellular fraction, 3-mycelia associated fraction.

These experiments confirmed that the cloned *fus*A* gene is expressed in *S. lividans*. As expected, the enzyme FusA* is located intracellularly. FusA* was only partially purified and used in the conversion of fusidic acid, testing the cofactors (NAD, NADPH, NADH, FAD) and reducing agent (DTT) (Fig. 3.53).

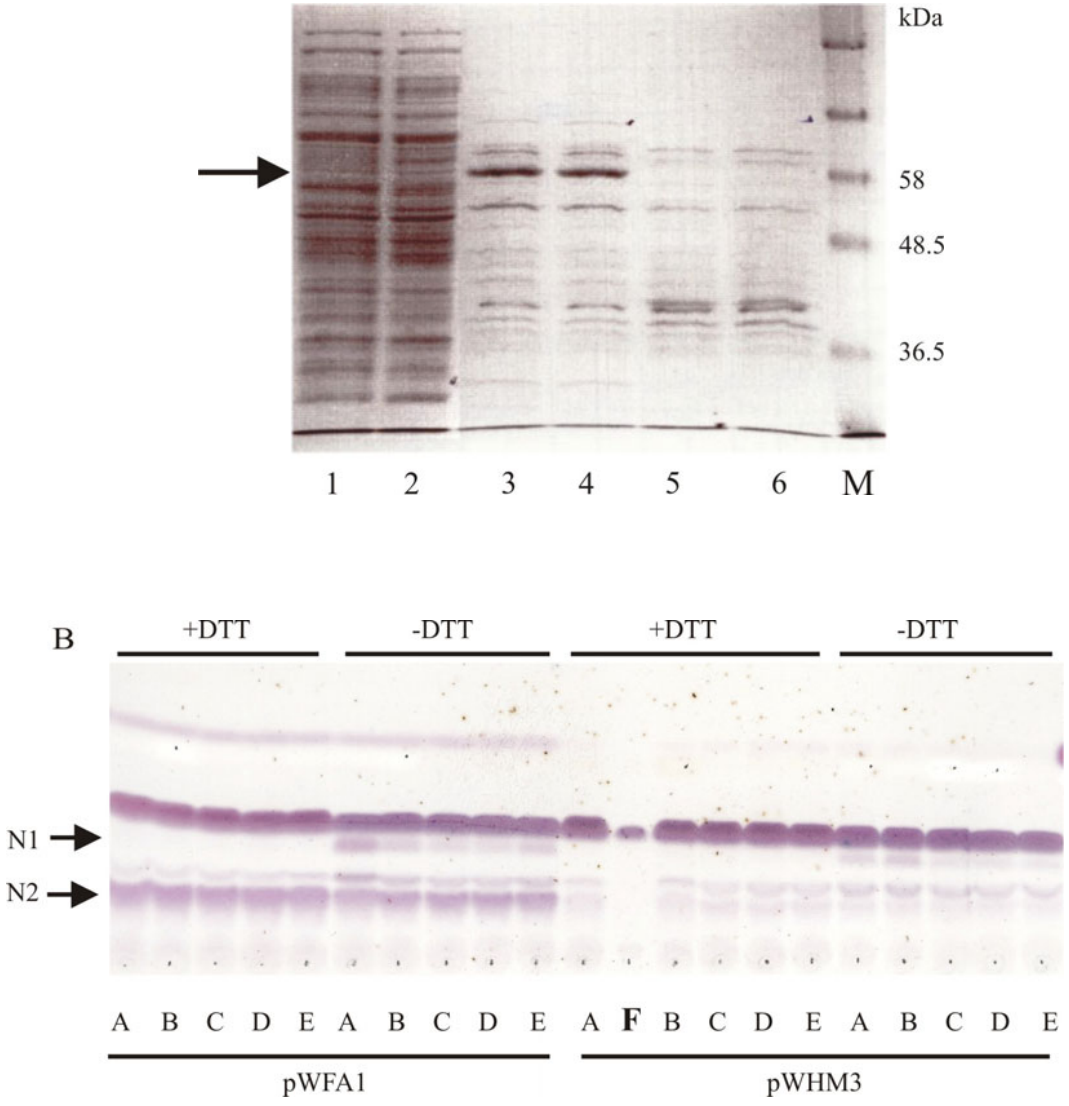


Figure 3.53 Enzymatic activity of partially purified proteins from Ni-NTA

A. Testing was done in the presence (lane 3, 5) or absence (lane 4, 6) of 1 mM DTT. The Ni-NTA purification was done as described above except that eluting step was omitted. The matrix with bound proteins was directly used for enzyme reaction and for SDS-PAGE analysis. The arrow shows the size of FusA*. M-protein marker 7B, *S. lividans* pWHM3 control (lanes 1, 5, 6), *S. lividans* pWFA1 (lanes 2, 3, 4). Soluble intracellular fraction (lanes 1, 2), proteins eluted from Ni-NTA (lanes from 3 to 6).

B. Test for the conversion of fusidic acid using the enriched proteins of *S. lividans* pWFA1 (8 A lane 4) and the control *S. lividans* pWHM3 (8A lane 6) in the presence (+) or absence (-) of 1 mM DTT and with the addition of the following cofactors (1 mM): A-NAD, B-NADP, C-NADPH, D-NADH, E-FAD. The assay was done in

buffer: 50 mM Na-phosphate pH 7, 37°C/overnight, F- fusidic acid. The arrows show the products: N1 (Rf 0.38) and N2 (Rf 0.24).

TLC analysis (Fig. 3.53B) showed that there is a significant difference in fusidic acid conversion between partially purified proteins from *S. lividans* pWFA1 and the control *S. lividans* pWHM3. Substance N1 was synthesized under both reducing and non-reducing conditions and it was independent from the cofactors used. Synthesis of the substance N2 depends on the oxidized type of the cofactor, preferably NAD (pWFA1-A) and to a lesser extent on FAD (pWFA1-E). The binding affinity of FusA* to the Ni-NTA in the presence of the reducing agent (1 mM DTT) was not affected.

To further purify FusA*, binding to the Anion exchange DEAE chromatography was tested. DEAE binding analysis showed that FusA* binds efficiently at pH 7 and 8, and under these conditions it was eluted from the matrix using NaCl in concentration between 100 and 300 mM (Fig. 3.54).

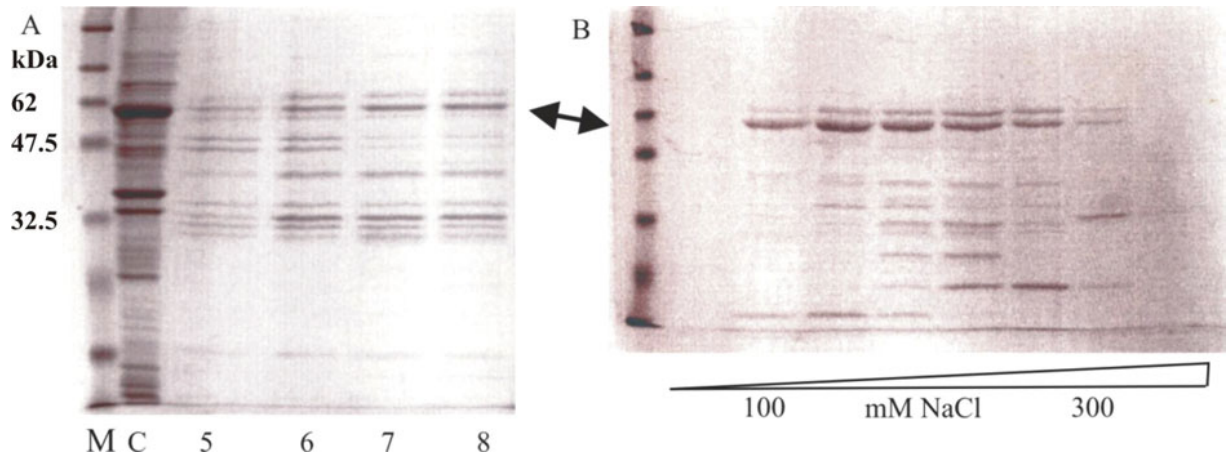


Figure 3.54 Optimization of the conditions for FusA*-isolation using Anion exchange DEAE chromatography

The analysis was done in 50 mM Na-phosphate buffer, pH from 5 to 8 (**A**). Protein fractions eluted from DEAE were individually subjected to the Ni-NTA affinity chromatography. After binding (10 mM imidazole) and washing (25 mM imidazole), remaining proteins were eluted (250 mM imidazole) and the aliquots were analysed by SDS-PAGE. **A** Ni-NTA purification of FusA* from DEAE batch pH from 5 to 8. M protein marker 8S, C-control for FusA*. **B** Ni-NTA purification of FusA* from DEAE pH 8, linear gradient 0-500 mM. NaCl. The arrow shows the size of FusA*.

Based on these results, Anion exchange DEAE chromatography was chosen as a purification step prior Ni-NTA affinity chromatography. Two proteins (apparent size of ~60 kDa and ~35 kDa) were purified (Fig. 3.55).

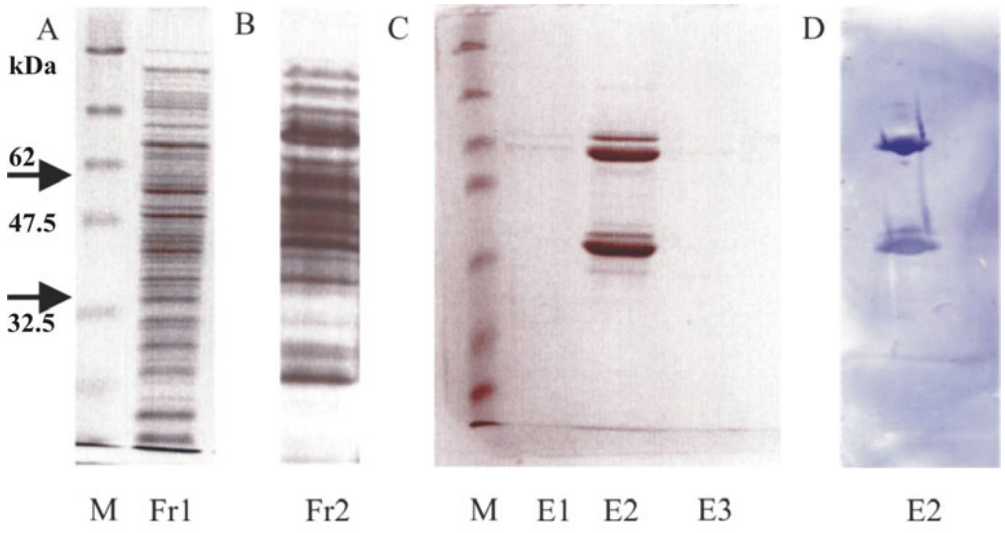


Figure 3.55 Purification of FusA* and its confirmation by coupled reaction using alkaline phosphatase linked to Ni-NTA

The cleared supernatant (Fr1), obtained after sonication of mycelia and centrifugation was applied directly on an anion exchange DEAE HR 10/10 column and proteins were eluted with gradient of 0-50% NaCl (10 CV). Fractions from 10-30% NaCl were pooled together (Fr2), adjusted the salt concentration (500 mM NaCl) and imidazole (25 mM) and subjected to the batch Ni-NTA affinity chromatography (1 h/ 4^{o}C). Bound proteins were washed (30 mM imidazole) and eluted stepwise with 250 mM imidazole (E1-E3) (see Materials and methods).
A, B, C- SDS-PAGE**:** M-protein marker 8S, **A**- Fr1- cleared supernatant of the sonicated mycelia of *S. lividans* pWFA1 transformant, **B**- Fr2-DEAE fraction, **C**- E1 to E3- Ni-NTA chromatography eluates of FusA* A. The arrows show the size of FusA* and its truncated form. **D-** Western blot of the eluate 2 (C-E2) from Ni-NTA chromatography using the alkaline phosphatase coupled with the Ni-NTA.

The positive results from Western blot analysis using alkaline phosphatase coupled with Ni-NTA confirmed that both proteins contained a His-tag. The larger band (about 60 kDa) corresponds to the full size of FusA*. Thus the smaller protein is likely a proteolytic product of FusA*. The fraction containing these two proteins was used in the in vitro assay using the substrate fusidic acid or lactone derivative (Fig. 3.56).

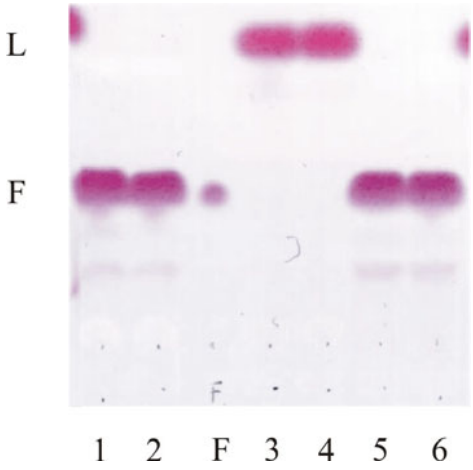


Figure 3.56 TLC analysis of enzyme reaction using FusA*

The enzyme assay contained the enzyme FusA* (Fig.10-C/E2) in 50 mM Na phosphate buffer pH 6 (reaction 1, 3, 5) or pH 8 (reaction 2, 4, 6) containing cofactor mix (1 mM NAD, NADPH, NADH, FAD, NADP) at 37°C/ overnight. TLC analysis was done as described in Materials and methods. **1, 2**- fusidic acid **3, 4-** lactone derivative, **5, 6-** control (without enzyme + fusidic acid). F- fusidic acid.

The TLC analysis showed that neither fusidic acid, nor the lactone derivative was converted. These results suggested that there are several possibilities that could explain the observed fusidic acid conversion:

1. FusA* needs additional factor(s) for the converting activity.
2. Either the wild type FusA instead of FusA* or another protein converts fusidic acid, which both may interact with FusA*.

A protein homology search revealed that the protein FusA is homologous with the protein AidB from *E. coli*. This enzyme has an isovaleryl-CoA dehydrogenase activity, converting isovaleryl-CoA to ß-methylcrotonyl-CoA. (Zhang et al. 1999). Therefore FusA* was also tested for this activity (Fig. 3.57).

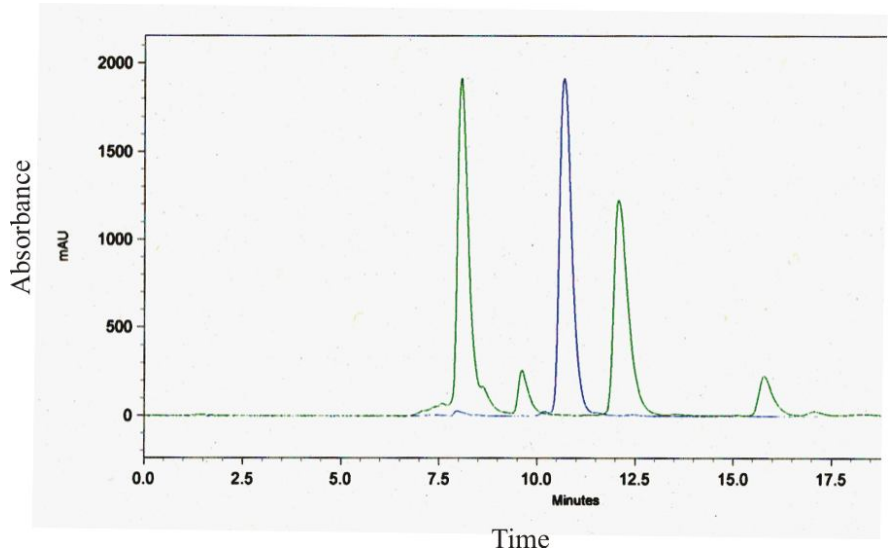


Figure 3.57 RP-HPLC analysis of isovaleryl-CoA activity of FusA* from *S. lividans* pWFA1

The enzyme assay (100 µl) contained: 1 mM isovaleryl-CoA, 1.33 mM PMS (phenazine methosulphate), 0.4 mM FAD in 100 mM Na-phosphate buffer pH 8. Reaction was done at 37°C/ overnight. An aliquot of the enzyme assay was mixed 1:1 with the running buffer (50 mM K-phosphate buffer pH 5.7: methanol = 50:50) and applied on the C18 RP-HPLC HyPurity Elite column. Substances from the assay were separated (depicted in **green**) with the following retention times (Tr): Tr (FAD) 8.2 min., Tr (PMS) 9.6 min., Tr. (isovaleryl-CoA) 12.1 min., Tr (degradation product) 16 min. For the comparison, a peak of ß-methylcrotonyl-CoA (depicted in **blue**, Tr 10.7 min.) is aligned with the chromatograph.

Under the described conditions, FusA* did not show isovaleryl-CoA dehydrogenase activity. The observed substance with the retention time Tr 16 min., also appeared in the control reaction (data not shown) and is a result of the non-enzymatic degradation of the phenazine methosulphate.

9. Expression of the cloned *fus*G gene and purification of its product

9.1 Expression of the *fus*G* gene in *E. coli*

The expression strain *E. coli* BL21 (λDE3, pLysS) was transformed with the plasmid DNA pTFG2. Transformants were selected by their resistance against kanamycin and chloramphenicol and two of them were precultivated and induced with the 1 mM IPTG. The pattern of proteins gained from induced and uninduced *E. coli* pTFG2 transformants, 3 h after the induction, did not show a difference in the expected size of an extra band that would correspond to FusG* (Fig.3.58-lanes 5, 6 and 7). Purification of FusG* using affinity chromatography Ni-NTA showed, however, clearly that the induced strain in comparison with uninduced, produced a protein whose molecular weight (MW) matches to that of FusG* (Fig.3.58 lane 1-4).

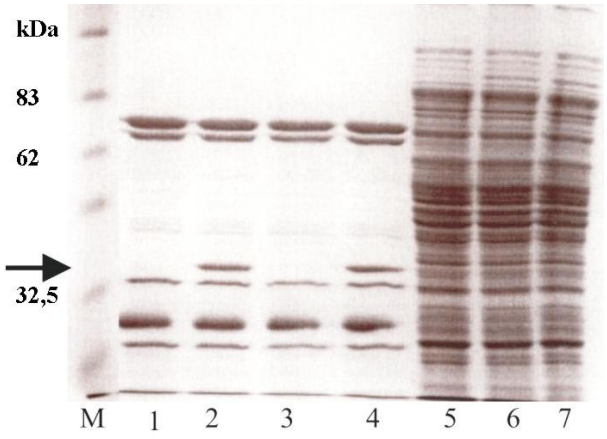


Figure 3.58 Ni-NTA partial purification of FusG*

The *E. coli* BL21 (λDE3, pLysS) transformants pTFG2-1 (lanes 1, 2, 5, 6) and pTFG2-2 (lanes 3, 4, 7) were propagated and induced under the standard conditions (see Materials and methods). As a control served the same strain but the induction was omited. After centrifugation, an aliquot of the pellet of the induced (lane 5, 7) and uninduced (lane 6) transformants were treated with 1% SDS and directly applied on the gel. The soluble intracellular fractions of induced and uninduced *E. coli* pTFG2 transformants were subjected to the Ni-NTA matrix (lanes 2, 4– induced and lanes 1, 3- uninduced). After binding (10 mM imidazole) and washing (15 mM imidazole), FusG-His tag (FusG*) was eluted using 250 mM imidazole (lanes 1-4). Proteins were separated by SDS-PAGE: M-protein marker 8S. The arrow shows the the size of FusG* .

Both fractions of the *E. coli* transformants pTFG2 (Fig. 3.58, lane 1- as a negative control and lane 2- containing the cloned *fus*G gene) were used to test, whether fusidic acid or the mixture of 16ß-OH derivative and lactone served as substrates (Fig. 3.59).

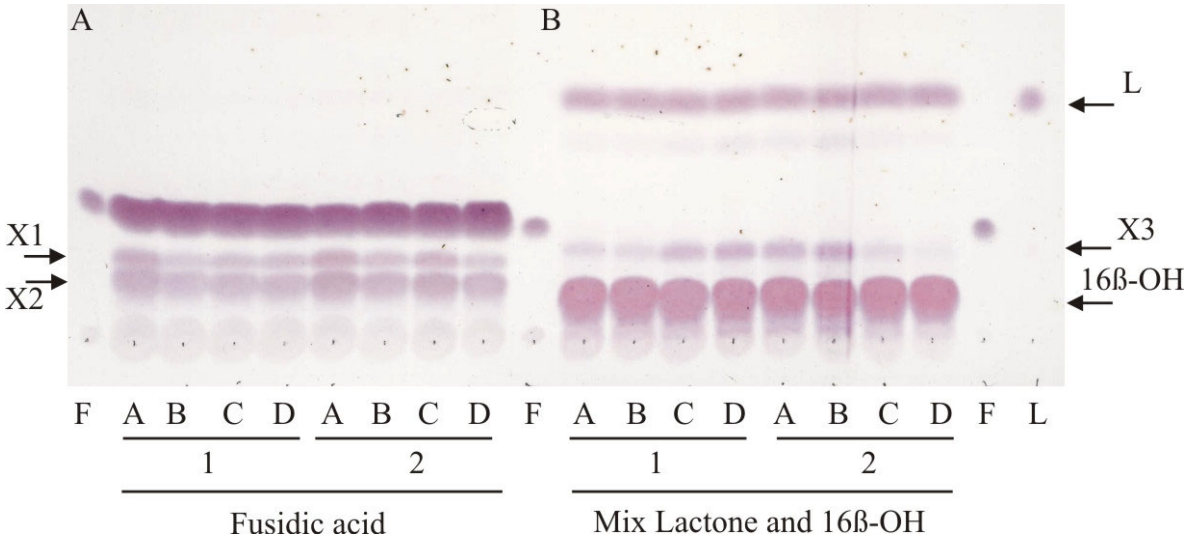


Figure 3.59 Enzymatic conversion of fusidic acid, 16ß-OH and lactone derivative by FusG*

TLC analysis of partially purified FusG* by Ni-NTA affinity chromatography (see Fig. 3.58). **1-** induced expression with IPTG (Fig. 1-lane 2) and **2**- not induced expression, (control**,** Fig. 1- lane 1). The enzyme reaction was done in 50 mM Na-phosphate buffer pH 8 at 37^{O}C/ overnight. Tested cofactors 1 mM: A- NAD B- NADH, C- NADPH, D- FAD. TLC analysis was done as described in Materials and methods. F- fusidic acid, L- lactone, 16ß-OH derivative, x1-3 synthesized products.

The fractions obtained from induced and uninduced *E. coli* pTFG2 transformants lead to identical conversion products either from fusidic acid or a mixture of the lactone and the 16ß-OH derivative. The products X1 (Rf 0.35), X2 (Rf 0.24) and X3 (Rf 0.37) were produced independently of the presence or absence of FusG*.

9.2 Expression of the *fus*G* gene in *S. lividans*

S. lividans pWFG1 transformants were cultivated as previously described. Different fractions from the liquid culture, supernatant and mycelia, were subjected to the Ni-NTA affinity chromatography in order to localize FusG* protein (FusG having His-tag at the C-terminus) (Fig. 3.60).

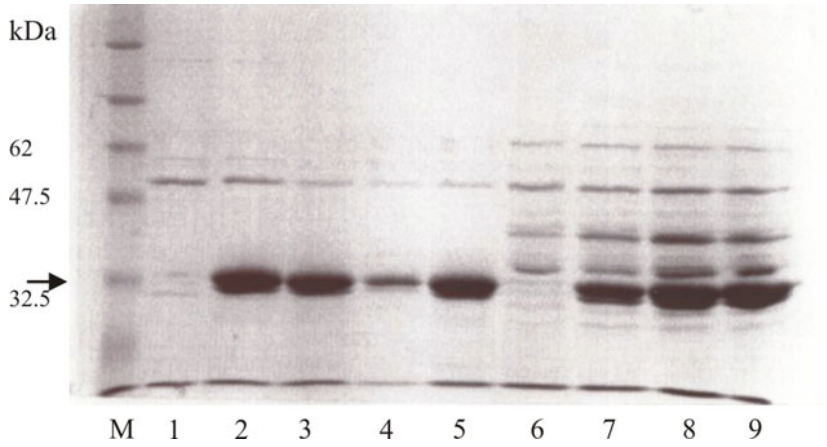


Figure 3.60 Localization of FusG* protein

Purification of FusG* from the culture filtrate (lanes 1-5) and the mycelia (lanes 6-9) of the different *S. lividans* pWFG1 transformants (lanes 2-5 and 7-9) and the control strain *S. lividans* harbouring pWHM3 (strain without the cloned *fus*G* gene: lane 1 and 6). Proteins from the culture filtrate were obtained after $(NH_4)_2SO_4$ precipitation (90% w/v), dialyzed (50 mM Na-phosphate buffer, 300 mM NaCl, pH 8) and subjected to the Ni-NTA affinity chromatography in the presence of 15 mM imidazole. Soluble intracellular fraction gained from sonicated mycelia was cleared from the cell debris and directly subjected to the Ni-NTA affinity chromatography. All fractions were washed with 35 mM imidazole and subsequently residual proteins were released with 250 mM imidazole. Aliquots of purified proteins were analyzed by SDS-PAGE. M- protein marker 8S. The arrow shows the size of the expected FusG*.

The gel analysis showed that FusG* is present in equal amounts in the culture filtrate as well as intracelullarly. In order to purify FusG* to homogeneity from both fractions, the purification procedure employing Ni- NTA affinity chromatography was further optimized (Fig 3.61).

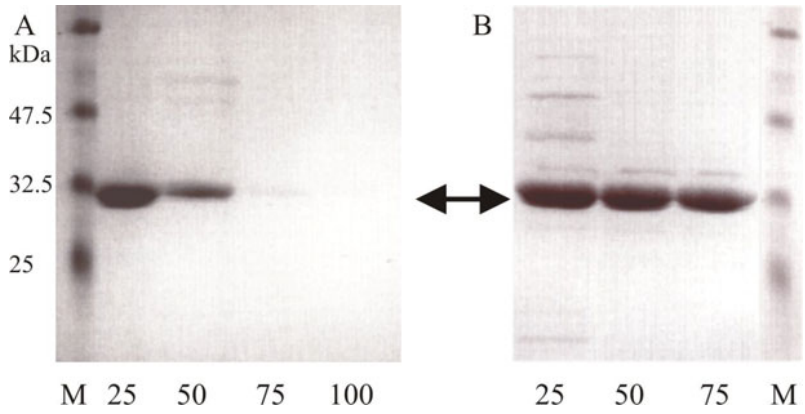


Figure 3.61 Ni-NTA purification of FusG* from the *S. lividans* pWFG1 transformant

The pWFG1 transformant was grown as described in Materials and methods. The proteins from the culture filtrate (**A**) gained after centrifugation were precipitated $(NH_4)_2SO_4$ (90% w/v), dialyzed (50 mM Na-phosphate buffer pH 8) and in the presence of 500 mM NaCl and 25 mM imidazole bound to the Ni-NTA matrix (1 h/ 4°C). The matrix was gradually washed with the indicated concentrations of imidazole (25, 50, 75, and 100 mM) and matrix-bound-proteins were directly analyzed by SDS- PAGE. The soluble intracellular proteins (**B**) were gained after sonication of mycelia followed by centrifugation, mixed with the Ni-NTA matrix and further purified under the same conditions as previously described for the proteins from the culture filtrate (see Materials and methods). M-protein marker 8S. The arrow shows the the size of FusG*.

A single protein band in the expected size of FusG* was purified from the culture filtrate of a *S. lividans* pWFG1 transformant. The cytoplasmatic fraction (B) contained one dominant protein with the expected size of FusG* and a minor amount of a protein, whose apparent molecular weight was slightly larger. Both fractions were further used in testing for the conversion of fusidic acid and the lactone derivative (Fig. 3.62).

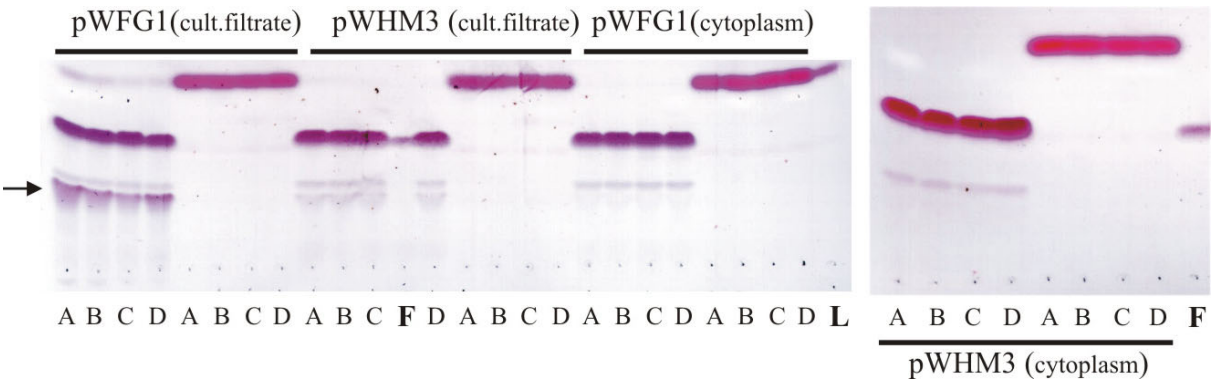


Figure 3.62 TLC analysis of enzyme reaction using FusG*

FusG* which was purified from the culture filtrate (Fig. 3.61A-25 mM) or the mycelia (Fig. 3.61B-75 mM) of *S. lividans* pWFG1 transformant was used in TLC analysis. Concentrated protein samples from the mycelia and the culture filtrate of the control strain *S. lividans* containing pWHM3 were prepared in the same way as FusG*. The enzyme assay consists of 50 mM Na-phosphate buffer pH 7 containing 5 µg substrate (fusidic acid or lactone), 1 mM cofactors: A-NAD, B-NADPH, C-FAD, D-without cofactor at 37°C/ overnight, **F-** fusidic acid **L-** lactone. The arrow shows the product of the conversion (Rf 0.24).

Purified FusG* gained either from the culture filtrate or the mycelia did not convert the lactone derivative. Some conversion of fusidic acid, independent of the used cofactor, occurred by FusG* isolated only from the culture filtrate. It was shown that the native FusG (see Fig. 3.24) is able to convert both substances A and the B, therefore FusG* gained from the culture filtrate of the *S. lividans* was used to confirm the obtained results (Fig. 3.63).

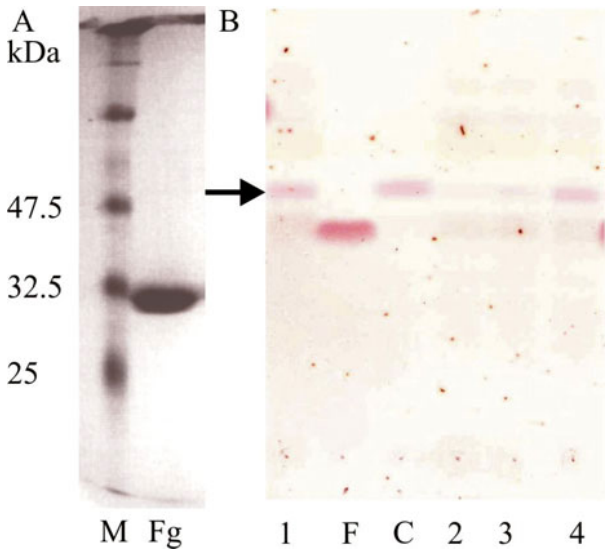


Figure 3.63 Enzymatic conversion of the substances A/B using the enzyme FusG*

FusG* (A-Fg) was purified from the culture filtrate of the *S. lividans* pWFG1 (see Fig. 3.61).

A-SDS-PAGE of Ni-NTA purified FusG* (Fg). M-protein marker 8S. **B** TLC analysis of enzymatic conversion of the RP-HPLC purified substance A/B, using cofactors (1 mM): 1-NAD, 2-NADH, 3-NADPH, 4-NADP, C-control reaction containing substances A/B and without enzyme. The enzyme reaction was done in 50 mM Na-phosphate buffer pH 7 at 37°C/ overnight. F- fusidic acid. The arrow shows the mixed substrates used in reaction and its disappearance in 2 and 3.

Although the amounts of the substrates in the assays were on the limit of detection, when compared with the control, it could be seen the conversion of the substrate when the NAD(P)H were used

The enzyme FusG* was tested for isovaleryl-CoA dehydrogenase activity (Fig.3.64).

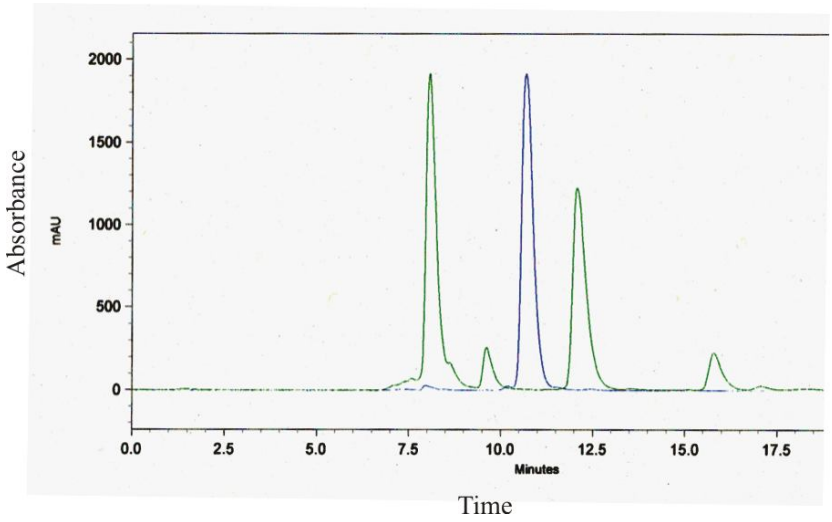


Figure 3.64 RP-HPLC analysis of isovaleryl-CoA activity of FusG*

Enzyme assay (100 µl) contained: 1 mM isovaleryl-CoA, 1.33 mM PMS, 0.4 mM FAD in 100 mM Na-phosphate buffer pH 8. Reaction was done at 37°C/ overnight with FusG* purified from culture filtrate and the cytoplasm of the *S. lividans* pWFG1. The aliquot of the enzyme assay was mixed 1:1 with the running buffer (50 mM K-phosphate buffer pH 5.7: methanol = 50:50) and applied on the C18 RP-HPLC, HyPurity Elite. Substances from the assay were separated (depicted in **green**) with the following retention times (Tr): Tr (FAD) 8.2 min., Tr (PMS) 9.6 min., Tr (isovaleryl-CoA) 12.1 min., Tr (degradation product) 16 min. For the comparison, a peak of ß-methylcrotonyl-CoA (depicted in **blue**, Tr 10.7 min.) is aligned with the chromatograph.

FusG* which originated either from the culture filtrate or the cytoplasm was tested for the isovaleryl-CoA dehydrogenase activity. The assay confirmed the inability of the enzyme to convert the isovaleryl-CoA into the ß-methylcrotonyl-CoA. The observed new substance with the retention time Tr 16 min., which also appeared in the control reaction (reaction without the enzyme; data not shown) was a result of the non-enzymatic degradation of the PMS.

IV Discussion

1. *S. lividans* is capable of transforming the fusidic acid derivatives

As shown in a previous work, *S. lividans* when grown in the presence of the antibiotic fusidic acid, synthesizes and secretes the highly specific esterase FusH, which efficiently deacetylates the antibiotic giving two inactive products. One is an unstable 16ß-OH derivative (16ß-deacetylfusidic acid) which converts to lactone (16ß-deacetylfusidic acid lactone), (von Haar et al. 1991, 1995 and 1997). The activity of both substances against the *Staphylococcus aureus* is about 400 times less than that of fusidic acid (Godtfredsen et al. 1966).

During prolonged cultivation of *S. lividans* at least several new substances are found in the culture filtrate (Fig. 3.1 and 4.1). Their synthesis is independent of the nutrient conditions and is induced by fusidic acid. This was confirmed by the inability of *S. lividans* to convert the 16ß-OH derivative and the lactone when both were added without prior induction directly to the propagating culture. It is conceivable that the appearance of the converting enzymes, preceding with FusH is subtly regulated. Apparently the substances do not serve as nutrients since the growth of *S. lividans* in the minimal medium (containing fusidic acid but no carbon source) was impetuously retarded.

Among the several detected substances, which are related to fusidic acid particular attention was given to N1 (Rf 0.5), a product which is derived from the lactone by the action of FusB enzyme. Further analysis of the enzymatic reaction mixture by Reversed phase HPLC revealed that N1 consists of two substances, designated A and B, both of which have the same Rf 0.5. The amounts of A and B substances were insufficient for structural studies. Therefore, two substances (La and Lb) having the same Rf value (and presumably corresponding to A and B) were purified from the culture filtrate of *S. lividans*. The structural studies (Dr. Kasch and Dr. Liedke, Hans Knöll Institute Jena), showed that the substances have very similar structures, both related to the lactone. Their comparisons showed that the substance Lb contains an additional hydroxyl group at CH_3-28. The substance La differs from the lactone in the structure of the aliphatic side chain having a hydroxyl group at C-25 and the Δ^{23} double bond in *cis* configuration (Fig. 3.9 and 4.1).

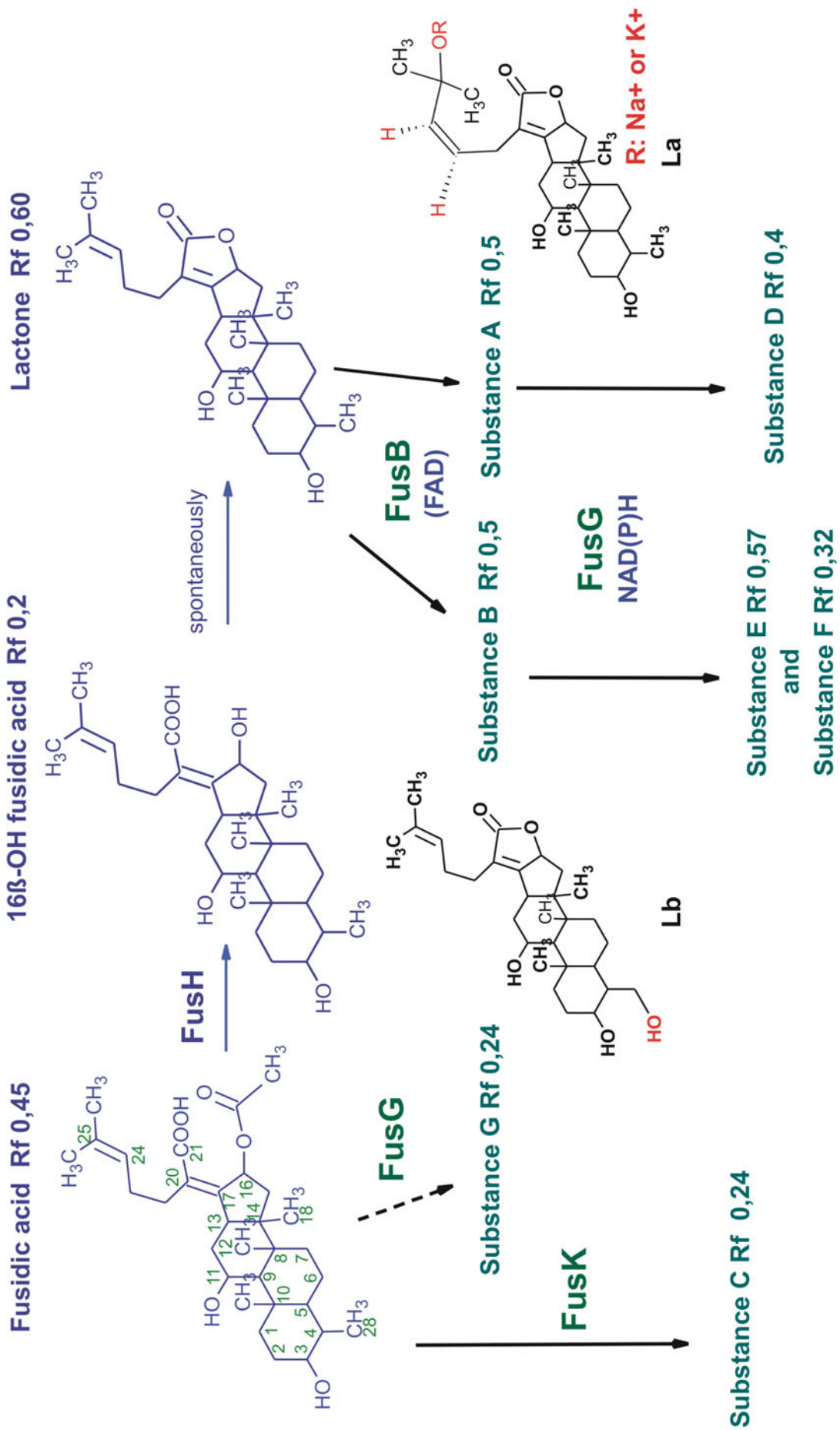

Fusidic acid Rf 0,45
16ß-OH fusidic acid Rf 0,2
Lactone Rf 0,60
FusH
spontaneously
FusG
FusB
(FAD)
FusG
NAD(P)H
FusK
Substance A Rf 0,5
Substance B Rf 0,5
Substance C Rf 0,24
Substance G Rf 0,24
Substance D Rf 0,4
Substance E Rf 0,57
and
Substance F Rf 0,32
La
Lb
R: Na+ or K+

Figure 4.1 Biotransformation of fusidic acid and its derivatives

The previously characterized biotransformation reactions (catalyzed by esterase FusH) of fusidic acid to lactone via 16ß-OH derivative is depicted in blue (von Haar et al. 1991, 1995 and 1997). The proposed metabolic routes catalyzed by the enzymes (FusG, FusB and FusK) are shown in green. The two characterized substances La and Lb which were purified from the liquid culture of *S. lividans* and possibly related to the substances A and B are shown in black. The conceivable conversion of fusidic acid by FusG is shown by a dotted arrow.

The fact that in the substance La the *cis*Δ^{23} double bond is in the ß position towards the 25-hydroxyl group implies the possible involvement of several different enzyme activities in its formation: hydration of a Δ^{24} double bond, α,ß-dehydrogenation (Δ^{23}) and *trans*/*cis* isomerization leading to the hydroxyl-enoyl derivative (Fig. 4.2).

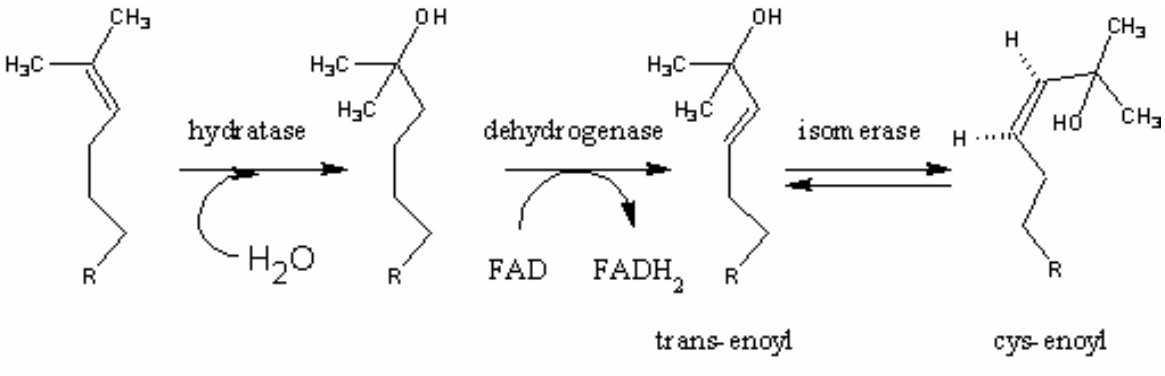


Figure 4.2 Possible sequence of reactions leading to substance La

For clarity only the side chain of the molecule where the reactions take place is depicted. R- represents the steroid nucleus of the lactone.

So far, modifications of a steroid side chain have been reported to be catalyzed either by enzymes belonging to cytochrome P450 superfamily, steroid dehydrogenases or enzymes involved in ß-oxidation. The enzyme reactions (Fig. 4.2) resemble the initial steps of ß-oxidation, being involved in the degradation of a large variety of fatty acids, metabolism of branched chain amino acids, synthesis of bile acids and degradation of different xenobiotics. The pathway comprises sequential steps of α,ß-dehydrogenation, hydration of the double bond, stereo-selective oxidation of a hydroxyl group and the cleavage of the keto-acyl thioester, which gradually leads to degradation of the side chain (Kim and Battaile 2002). The gained data suggest that FusG (30 kDa) and FusB are likely involved in the reactions corresponding to ß-oxidation. FusG is not able to convert the lactone, but rather the substances A and B (Rf 0.5) in the presence of NAD(P)H conferring three new products D, E

and F (Fig 4.1). The analysis of the deduced FusG protein showed similarity with many hydroxyacyl-CoA dehydrogenases from a variety of organisms, particularly with the putative FadB2 from *Mycobacterium tuberculosis* (59% identity) and the mitochondrial short chain L-3-hydroxyacyl-CoA dehydrogenase (L-3-scHAD) from human heart (41% identity) (Fig 4.3). The function of FadB2 (*M. tuberculosis*) is not known, but the mitochondrial human heart L-3-scHAD was shown to catalyze the oxidation of the hydroxyl group of L-3-hydroxyacyl-CoA to 3 ketoacyl-CoA in the presence of NAD (Barycki et al. 1999).

The mitochondrial human heart L-3-scHAD (34 kDa) is composed of two subdomains and assembles to a soluble homodimer. The N-domain (from amino acid residues 12 to 201), consists of a characteristic Rosmann fold with 8 stranded ß-sheets flanked by α-helices and an unusually large helix-turn-helix "tail" (ß2-α2-α3-ß3) containing numerous charged amino acids. The later structure (helix-turn-helix) is barely conserved in FusG, implying that its N-domain folding could be different from that of the human heart L-3-scHAD. Furthermore, in 3-scHAD a CoA moiety of the bound substrate is located in the vicinity of the helix-turn-helix "tail". This may suggest the relevance of the substrate activation by acetyl-CoA. The cofactor binding motif (Gly_{22}- Gly_{23}- Gly_{24}- Leu_{25}-Met_{26}-Gly_{27}) with the cofactor interacting residue Met_{26} (Barycki et al. 1999) is partially conserved in FusG (Gly_{23}→Cys and Leu_{25}→Gln). The C-domain (from 207 to 302 amino acid residue) of the L-3-scHAD consists primarily of α-helices, which are involved in subunit dimerization and binding of the substrates. Three conserved aspartic residues in this domain make hydrogen bonds with Tyr_{299} (Asp_{233}), Tyr_{301} (Asp_{251}) and a salt bridge with Lys_{293} (Asp_{256}). All these amino acids are also conserved in the FusG sequence, indicating a structural similarity of C-domain. This is additionally supported by the conserved Leu_{211} and Pro_{213} residues in the α10 helix which is responsible for hydrophobic interactions necessary for dimerization.

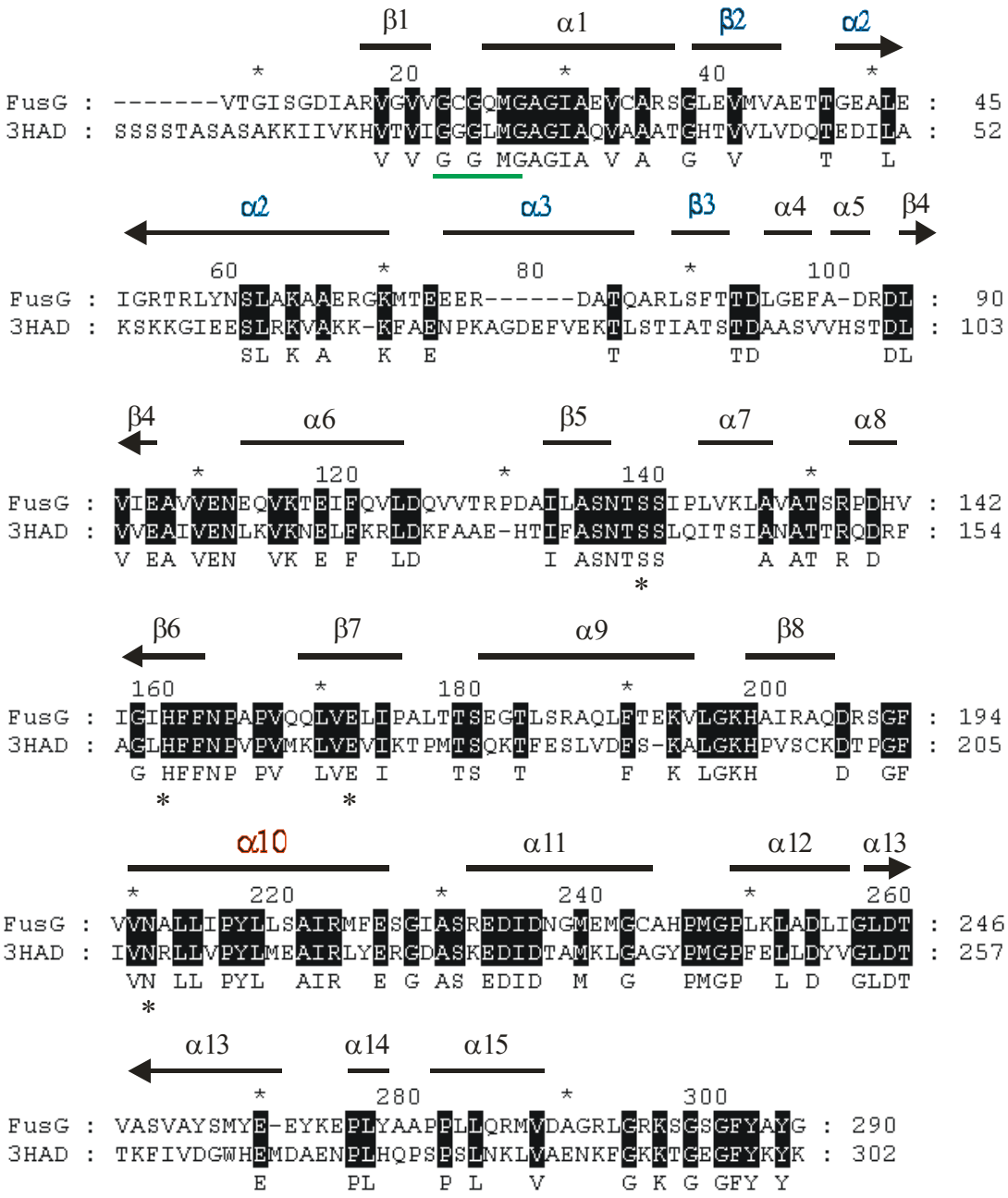


Figure 4.3 Protein sequence alignment of FusG (*S. lividans*) and mitochondrial human heart L-3-hydroxyacyl-CoA dehydrogenase

Identical amino acid residues are black shaded. Beneath the aligned sequences is the consensus sequence. Secondary structure (α-helix and ß-sheet) according to the described L-3-scHAD (shortened in the alignment 3HAD) from human heart is assigned to the alignment. Four amino acid residues creating catalytic site (His_{158},Glu_{170}, Ser_{137} and Asn_{208}) are signed with the asterix "*" under the consensus line, the Rosmann fold signature is underlined with the green, α10-helix responsible for dimerization is marked in red and unusual helix-turn-helix "tail" is in blue (Barycki et al. 1999).

The crystal structure of human heart L-3-scHAD was solved in its apoenzyme form, in the binary complex either with NAD^+/NADH or with 3-hydroxybutyryl-CoA and in the ternary complex with NAD^+ and acetoacetyl-CoA (Fig. 4.4) (Barycki et al. 1999 and 2000).

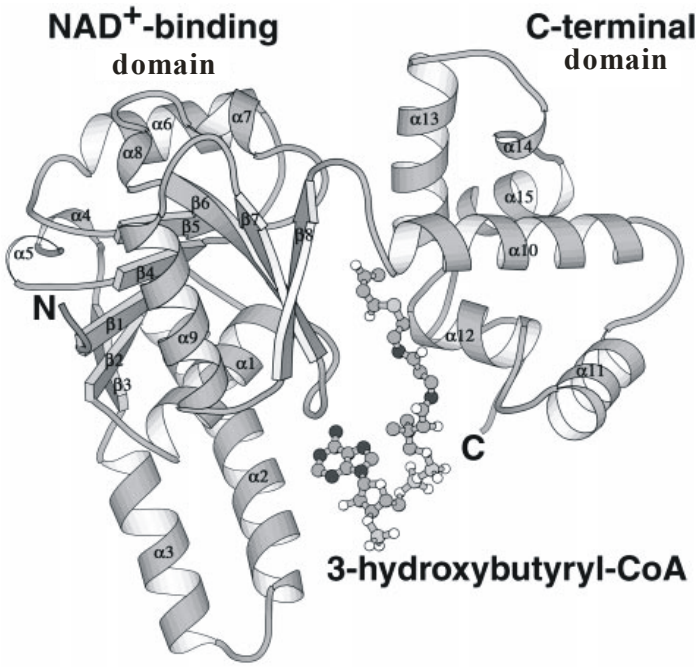


Figure 4.4 L-3-hydroxyacyl-CoA dehydrogenase complexed with the substrate 3-hydroxybutyryl-CoA
The ribbon diagram depicts the two-domain structure of an L-3-scHAD subunit, with the first 200 amino acids comprising the NAD-binding domain and the remaining residues comprising the C-terminal domain. The 3-hydroxybutyryl-CoA, shown in ball and stick binds within the cleft between these two domains. The adenine moiety of Coenzyme A is positioned adjacent to the helix-turn-helix tail (α2–α3) of the NAD-binding domain, and the acyl chain is within the enzyme active site (Barycki et al. 2000).

Molecular modelling and mutagenesis studies confirmed that His_{158} serves as a general acid/base in the catalytic mechanism of hydroxyacyl-CoA dehydrogenase, with catalysis facilitated by the presence of the Glu_{170} (He and Yang 1996, He et al. 1997, Barycki et al. 1999, 2000 and 2001). In the vicinity of these two residues there are Ser_{137} and Asn_{208} conserved, which both participate in the catalysis by assisting in the hydride-movement (Barycki et al. 1999). These four residues (His_{158}, Glu_{170}, Ser_{137} and Asn_{208}) are also present in FusG, implying that such reaction mechanism is likely preserved. FusG shows substrate specificity in converting the substances A and B, but not the lactone in the presence of reduced cofactors NAD(P)H (see Fig. 3.62 and 3.63). The basis for the substrate

discrimination is not clear, even if one assumes that structures of the substances La and Lb are identical to the substances A and B. Furthermore, it remains to be elucidated whether the observed weak conversion of fusidic acid by native FusG (see Fig. 3.23) is a cofactor independent reaction.

The L-3-scHAD is closely related to the α-subunit of the mitochondrial trifunctional protein (MTP), the α-subunit of FadAB complex (*E. coli*) and the peroxisomal multifunctional protein 1 (MFE1), both of which additionally exhibit hydratase, isomerase and thiolase activity towards the acyl-CoA substrates. A second peroxisomal multifunctional enzyme MFE2 catalyzes the same sequel of reactions in ß-oxidation of branched chain fatty acids and bile acid biosynthesis, which in both examples proceed via the D-isomer of 3-hydroxy-(acyl/steroid)-CoA (Russell 2003). Sequence analysis has revealed that MFE2 is identical to the 17ß-hydroxysteroid dehydrogenase type 4 (17ß-HSD-4) that is not related to the L-3-hydroxyacyl-CoA dehydrogenases (Breitling et al. 2001). In peroxisomes, MFE2 undergoes proteolytic cleavage giving rise to the soluble 17ß-HSD-4/D-3-hydroxyacyl-CoA dehydrogenase and the 2-enoyl-CoA hydratase-2 (Noubhani et al. 1996). The full length MFE2 and the truncated protein exhibit steroid/acyl-CoA dehydrogenase activity (Leenders et al. 1996). Hydroxysteroid dehydrogenases perform oxidation/reduction of various hydroxyl/oxo groups of steroids using the cofactors NAD(H) or NAD(P)H (Jez et al. 1997, Adamski and Jakob 2001). The dual mode of catalysis is related to the pseudo-symmetry of the steroid nucleus (performing reactions on opposite sides of the steroid molecule) and to the differences in the structure of the substrate. The later one may govern the type of the reaction, one side of the molecule being oxidized and the other one reduced (Gangloff et al. 2003). This is supported by the recent finding that the 17ß-hydroxysteroid dehydrogenase (type 10) exhibits multiple catalyses including 3α/7α/7ß/20ß/21-hydroxysteroid dehydrogenase catalytic activity (Shafqat et al. 2003). Recent findings show that the novel L-type of hydroxyacyl-CoA dehydrogenase (within mitochondria of the human brain) which lacks significant amino acid identity with the common L-3-hydroxyacyl-CoA dehydrogenases, catalyzes the oxidation and reduction of steroid hormones and various aliphatic alcohols in the presence of NAD(H) (He et al. 1999 and 2000).

Whether FusG comprises these features still remains unresolved, due to the limited amount of the available substrates A and B. Another interesting feature of FusG is related to its localization. FusG-His tag was found to be equally present in *S lividans* intracellularly as well as secreted. The analysis of deduced protein sequence did not confirm any potential peptide leader sequence that can explain its secretion. Despite the presence of an arginine rich

sequence (G-R-R-N-R-D-H) 30 amino acid residues from the first amino acid valine, the conserved sequences characteristic either for secretory (A-X-A) or twin arginine transport S/T-R-R-X-R-F-L-K are not found (Lammertyn and Anné 1998, Bentley et al. 2002, Schaerlaekens et al. 2001). Recently described genes encode a homologue of the known "single recognition particle" (SRP) pathway also in *S. lividans*, which is expected to be used in certain conditions (Palacín et al. 2003).

Partially purified FusB (~40 kDa and 30 kDa protein, see Fig. 3.17) converts in the presence of the cofactor FAD the lactone into the substances A and B (Rf 0.5) (see Fig. 4.1). The attempts to purify each of them resulted in the loss of the enzymatic activity. The aliphatic side chain of the lactone is structurally similar to the branched side chain of cholesterol, which is subjected to ß-oxidation in mammalian peroxisomes (Russell 2003). So far, the acyl-CoA dehydrogenase/ oxidase is the only known class of enzymes that converts steroids and its related substances using the cofactor FAD. The Δ^{24} double bond in the lactone could be a hindrance for the predicted reaction mediated by acyl-CoA oxidase (ACO). However it could be envisaged that the Δ^{24} double bond can be converted by an enoyl-CoA hydratase leading to the 25-hydroxyl lactone derivative (see Fig. 4.2). A related type of cholesterol modification is mediated by a mammalian microsomal 25-hydroxylase (Björkhem 1992, Russell 2003). Interestingly ß-oxidation of the bile acid precursor (trihydroxycoprostanoyl-CoA THCA-CoA) takes place in mammalian peroxisomes, wherein ACO and isomerase are soluble monofunctional enzymes. However the enoyl CoA hydratase and hydroxyacyl-CoA dehydrogenase are domains within the D-bifunctional protein. The purification procedure for the 30 kDa and 40 kDa proteins could have resulted in the loss of the predicted hydratase (see Fig. 4.2).

Among the ACO, only three of them dehydrogenate bile acid precursors (Schepers et al. 1990, Van Veldhoven et al. 1992 and 1996). The class of acyl-CoA dehydrogenase/ oxidase needs the cofactor FAD as a proton acceptor in order to provide the trans-2-enoyl CoA product (Ghisla et al. 1984). Most of the described isoenzymes of acyl-CoA dehydrogenases (ACDs) are soluble flavoprotein homotetramers having a ~43 kDa subunit complexed with one FAD molecule. (Kim and Battaile 2002, Kim and Miura 2004).

The pre-activation of fatty acids or precursors of bile acid requires an acyl-CoA synthetase or bile acid ligase prior the degradation of the aliphatic chain. The isolated lactone being converted by the partially acyl-CoA dehydrogenase FusB does not have a CoA moiety (see Fig 3.7B and 3.9). This may suggest an accessory enzyme, which may remove a formally present CoA moiety.

Native isoelectrofocusing of partially purified FusB revealed an interesting feature which is related to its exceptionally low isoelectric point pI ~3.5-4 and low catalytic pH optimum. A broader pI value may be related either to the inability to completely focus the protein (often characteristic of acidic proteins), or it may indicate the existence of the several isoenzymes slightly differing in the pI. These features differ from the characterized rat's (palmitoyl-ACO and THC-ACO) and plant's peroxisomal short chain ACO, all having pI ~8-9.5 (Hayashi et al. 1999, Schepers et al. 1990). FusB has highest activity at pH ~5. In comparison, other ACDs and ACOs have a pH range of 8-11 with an optimum of ~9 (Ghisla and Thorpe 2004, Hayashi et al. 1999, Vanhove et al. 1993). The α,ß-dehydrogenation is strongly pH dependent (Ghisla and Thorpe 2004), as similarly observed for FusB. The lack of FusB to convert the fusidic acid and the 16ß-OH derivative suggests that reduced polarity of the lactone ring with regard to the carboxyl and the C-16 hydroxyl group may play a crucial role in determining the substrate specificity. All these data imply that FusB could be quite distinct from the described acyl-CoA dehydrogenases.

A sequence analysis of the neighbouring DNA flanking the *fus*H gene (coding for the esterase FusH, von Haar 1997), revealed the presence of 3 open reading frames, including *fus*A which is located ~2 kb upstream of *fus*H and transcribed in the opposite direction. The gene codes for the putative ~59 kDa acyl-CoA dehydrogenase FusA (ACD) (Richter, Diploma Thesis 1999) and was therefore considered to determine the flavoprotein FusB. FusA belongs to the SDR superfamily whose members are able to convert a large variety of the substances (Jörnvall et al. 1995, Powell et al. 2000). Among many similar putative ACDs, two proteins having significant homology have emerged: FadE8 (55% identity) from *Mycobacterium tuberculosis* and AidB (45% identity) from *E. coli*. Although not yet characterized, FadE8 shows high homology with the acyl-CoA dehydrogenase FadE from *E. coli* (Nunn 1986, Cambell and Cronan 2002). On the other hand the Ada-inducible AidB protein is homologous to the mammalian isovaleryl-CoA dehydrogenase (ivACD), exhibiting isovaleryl dehydrogenase activity and probably it is involved in inactivation of nitrosoguanidines or their intermediates produced during metabolic detoxification (Fig 4.5), (Landini et al. 1994, Landini and Volkert 1995).

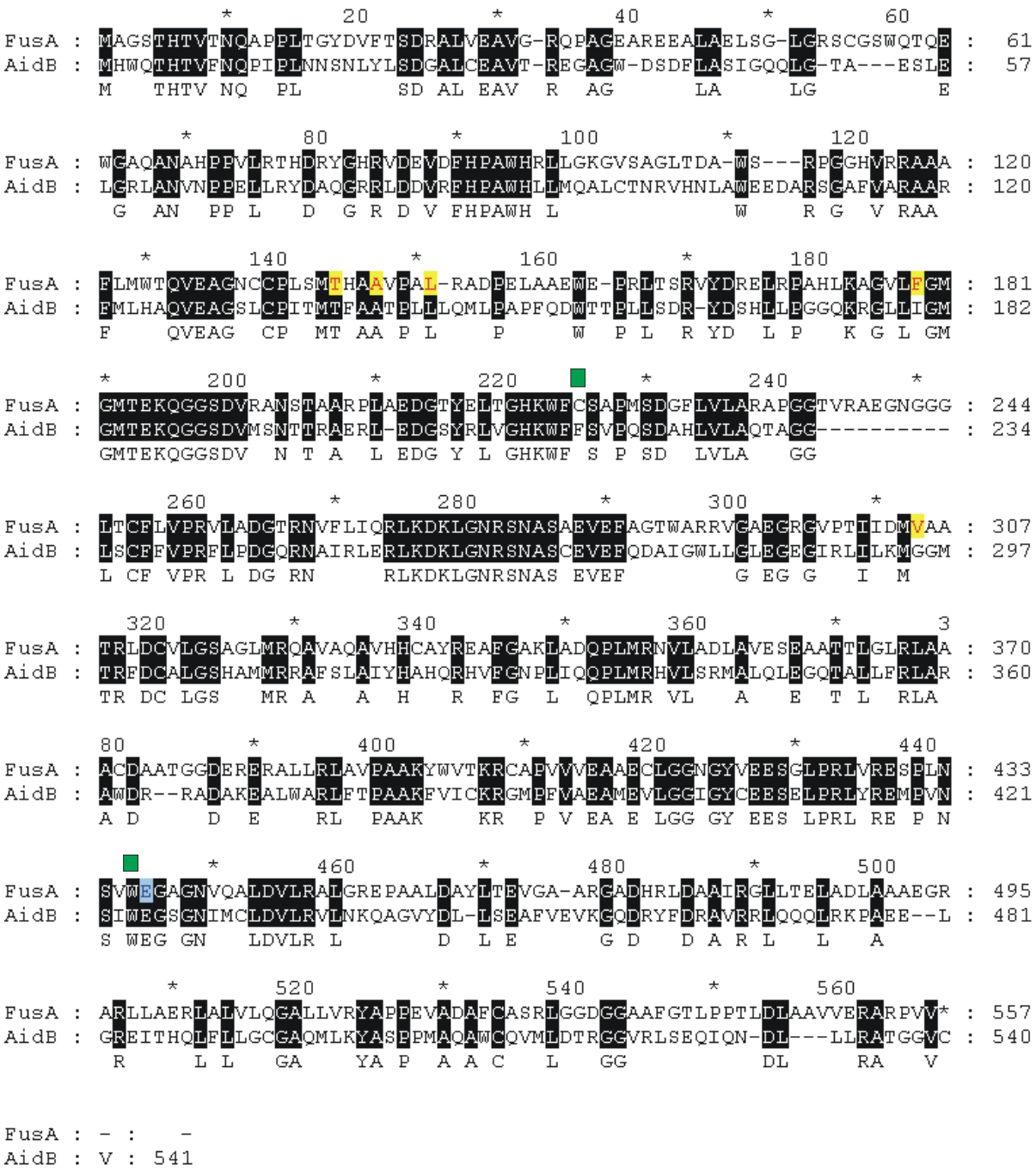


Figure 4.5 Protein sequence alignment of FusA (*S. lividans*) and AidB (*E. coli*)

Identical amino acid residues are black shaded. Beneath the aligned sequences is consensus sequence. Catalytic site glutamate is blue shaded. Conserved amino acid residues in FusA sequence, lining the substrate cavity are yellow shaded and non conserved residues are depicted with the green box above (Battaile et al. 2002).

Exposure of the *E. coli* to DNA-methylating substances triggers the process called the adaptive response in which the Ada protein (methyltransferase) plays a dual role, being both a DNA repair protein and a transcription activator (Landini and Busby 1999). The fact that the *aid*B gene is also induced during stationary phase in an RpoS-dependent manner in the presence of endogenous DNA-alkylating substances reflects its likely role in detoxification (Landini and Volkert 2000). The deduced AidB shares a large number of the identical amino acid residues throughout the whole protein, including the catalytic residue Glu_{437} as well as amino acids creating a substrate binding motif with deduced protein FusA (Fig. 4.5). According to the classification of Krasko and co-workers (1998) characteristic signatures of the "Glu-Gly class "-ACD/ACO enzymes can be found in FusA (Richter, Diploma Thesis 1999). FusA-His tag (FusA*), localized only intracellularly in *S. lividans* lacks the isovaleryl-CoA activity, which is a characteristic feature of AidB (Landini et al. 1994). In the course of purifying FusA* from *S. lividans* a ~35 kDa truncated form of FusA* was gained. Such a phenomenon was already observed with the esterase FusH (see Fig. 3.3 and 3.4) and with the mammalian 17ß-hydroxysteroid dehydrogenases, for which cleavage is a part of posttranslational modification (Leenders et al. 1996). Furthermore, FusA* (either expressed in *S. lividans* or *E. coli*) failed to convert fusidic acid and the lactone, indicating that it may need auxiliary enzyme(s) similar to a putative acyl-CoA dehydrogenase FusB (Fig. 3.50 and 3.56).

The possible role of FusA in biotransformation was indirectly shown by analysis of the 32 kDa FusK that selectively interacts with FusA* and is able to convert fusidic acid to the substance C (Rf 0.24) (see Fig 4.1). The conversion is independent of the tested cofactors, having a pH optimum from 6 to 9 and a temperature optimum between 37°C and 45°C. Immunodetection using the *anti*-FusH antibodies confirmed that both FusA* and FusK are not closely related to FusH. Accordingly, it is conceivable that FusK could be a part of the "intracellular defence" against fusidic acid.

The additionally proposed enzyme reactions: hydration and isomerization, which may contribute to synthesis of the La substance (see Fig 4.2) can be catalyzed by a type of enoyl-CoA hydratase which exists in mammalilan peroxisomes as a part of the L- and D-bifunctional proteins and soluble monofunctional Δ^3,Δ^2 enoyl-CoA isomerase (Qin et al. 1997 and 2000, Baes et al. 2000, Zhang et al. 2002). In prokaryotes (*E. coli*), related enzyme reactions include the FadAB multienzyme complex, which consists of the $\alpha_2ß_2$ subunits (α-78 kDa, ß-42 kDa) (Pawar and Shulz 1981). The isomerase and the epimerase activities are considered as auxiliary, necessary to couple degradation of the unsaturated fatty acids with the main degradation route (Spratt et al. 1984). The hydratase catalyzes reversible *syn*

addition of water to the Δ^2 (2E) enoyl derivative giving rise to (S)-3-hydroxyacyl-CoA (*E. coli* and mitochondria), or additionally (R)-3- stereoisomer (peroxisomes). This catalysis is closely related to the isomerase reaction of shifting a double bond either in *trans* or *cis* to the *trans*-C2 configuration (Fig. 4.5) (Willadsen and Eggerer 1975, Kiema et al. 1999). In the case of FusB one single enzyme may catalyze both reactions. Other enzymes of the hydratase/ isomerase superfamily have been reported to contain such dual catalytic activities like the soluble mitochondrial trans-enoyl hydratase-1 (ECH1), which has a intrinsic Δ^3,Δ^2 enoyl-CoA isomerase activity (Hiltunen et al. 2003, Kiema et al. 2002). Both reactions (hydration and isomerization) take place in a broad pH range (6-10) with the optimum at ~8 (Palosaari et al. 1991, Kiema et al. 1999).

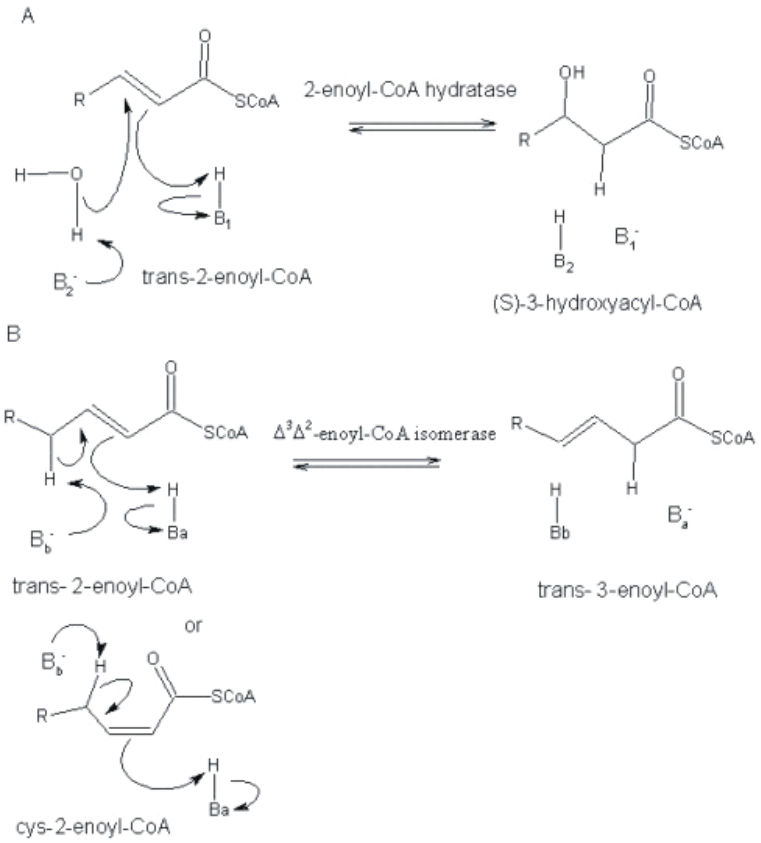
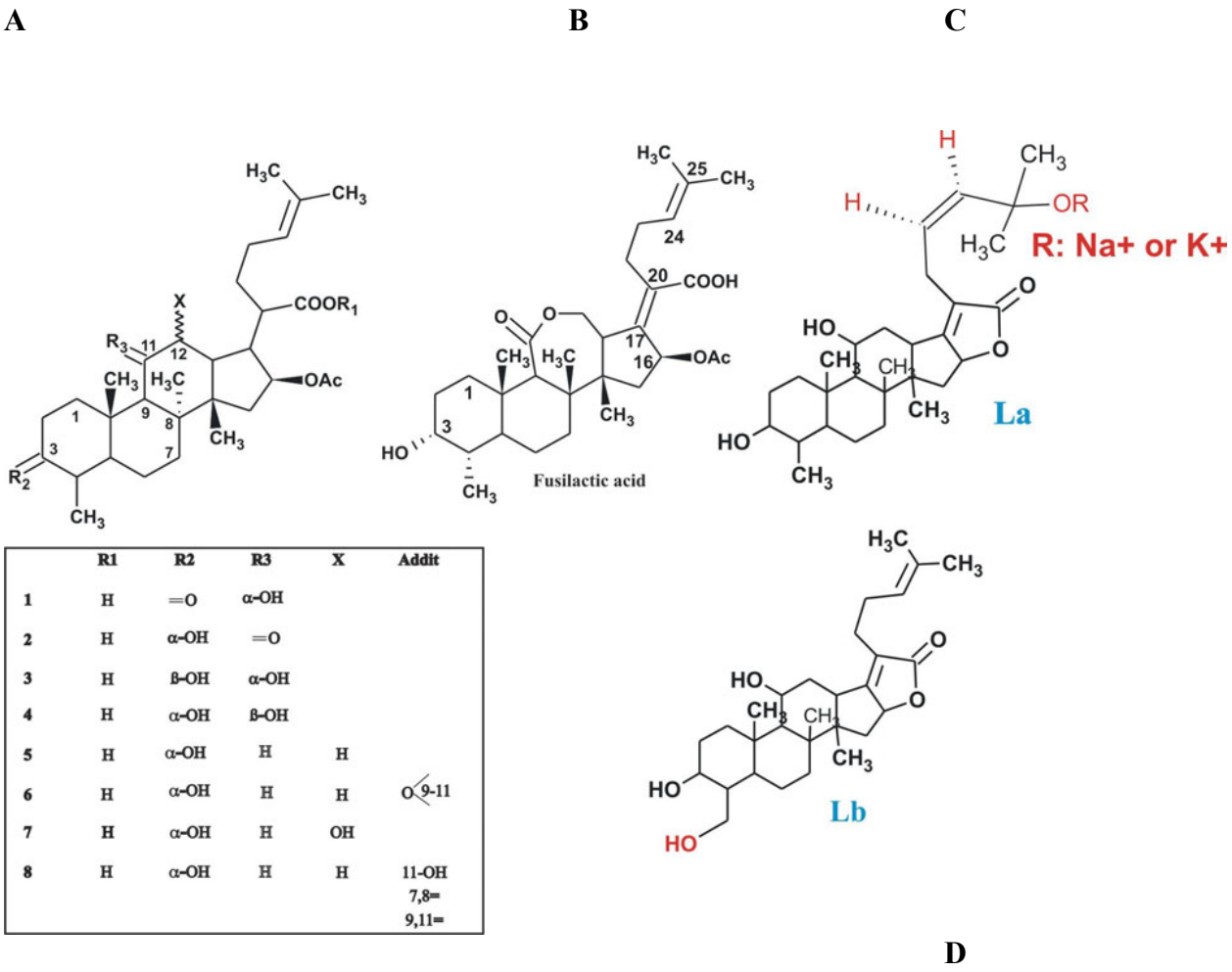


Figure 4.5 Reactions carried out by 2-enoyl-CoA hydratase 1 (A) and Δ^3 Δ^2-enoyl-CoA isomerase (B)

Acids $H\text{-}B_1$ and $H\text{-}B_a$ are Glu-164 in enoyl-coA hydratase and its equivalent in enoyl-CoA isomerase respectively. Base B_2 in the case of hydratase is Glu-144 and B_b^- for the enoyl-CoA isomerase is unknown

The purified substance Lb contains a hydroxylated methyl group at CH_3-28, which could be also a result of a reaction catalyzed by cytochrome P450. The cytochrome P450s are heme containing monooxygenases, that mostly hydroxylate a large variety of substrates using the cofactor NADPH and molecular oxygen (Miles et al. 2000, Reichhart and Feyereisen 2000). Genome sequencing of *S. coelicolor* A3(2) revealed 18 putative genes coding for P450 enzymes including the *cyp*51 gene, which codes for enzyme 14α-demethylase (Bentley et al. 2002, Lamb et al. 2002). It was shown that in fungi and mammals, a homologous enzyme successively converts 14-methylated sterols in three monooxygenation steps, resulting in elimination of formic acid and introduction of the Δ^{14} double bond (Shyadehi et al. 1996). Accordingly, the substance Lb could be the first intermediate in a similar reaction which at the end may lead to dealkylation and introduction of the Δ^4 double bond. A modification of the structurally related bile acids was described in *Eubacterium* sp., a prokaryote inhabiting the human intestinal bacterium It contains ~12 kb bile acid-inducible operon ("*bai*-operon") encoding 9 gene products, which are involved in the steroid ring modifications (so-called 7α-dehydroxylation pathway) (Hylemon et al. 1980 and 1991, Batta et al. 1990, Ye et al. 1999).
Fungus *Fusidium coccineum* produces the antibiotic fusidic acid. The key intermediates of biosynthetic pathway have been identified from the culture filtrate. Some of them were considered as a substrates for further modifications (Fig. 4.7) (Godtfredsen et al. 1979); their enzymatic reactions have been not determined. Like *S. lividans*, *F. coccineum* produces 16ß-OH derivative of fusidic acid, but not corresponding the lactone. Furthermore, the fact that none of the characterized metabolites has a modification on the aliphatic side chain, suggests that the biotransformation pathway in *S. lividans* might diverge from the one in *F. coccineum* in the early steps.



	R1	R2	R3	X	Addit
1	H	=O	α-OH		
2	H	α-OH	=O		
3	H	β-OH	α-OH		
4	H	α-OH	β-OH		
5	H	α-OH	H	H	
6	H	α-OH	H	H	O 9-11
7	H	α-OH	H	OH	
8	H	α-OH	H	H	11-OH 7,8= 9,11=

Figure 4.7 Cometabolites of fusidic acid isolated from the culture filtrate of fungus *Fusidium coccineum*
Fungal cometabolites (A and B) of fusidic acid in comparison with the newly isolated cometabolites La and Lb from the strain *S. lividans* (C and D).

The dermatophyte *Epidermophyton flocosum*, another producer of fusidic acid also accumulates mono- and additionally diketo-derivatives (3-keto fusidic acid, Fig. 4.7, substance 1; and 3,11-diketo fusidic acid) (Perry et al. 1983). The lactone and its inactive derivative 7α-OH lactone, as well as other so far poorely characterized derivatives were gained from the fusidic acid resistant plant pathogen *Nocardia brasiliensis* (Harada et al. 1999).

The analysis of chemically modified derivatives of fusidic acid led to the conclusion, that the side chain itself with the related Δ^{24} double bond is not important for the antibiotic activity. However the orientation of the C-20 carboxyl group towards the side chain in respect to the Δ^{17} double bond plays a significant role. That was confirmed by the finding that only the stereoisomer (17S, 20S), which retained the same activity as fusidic acid, contains the side

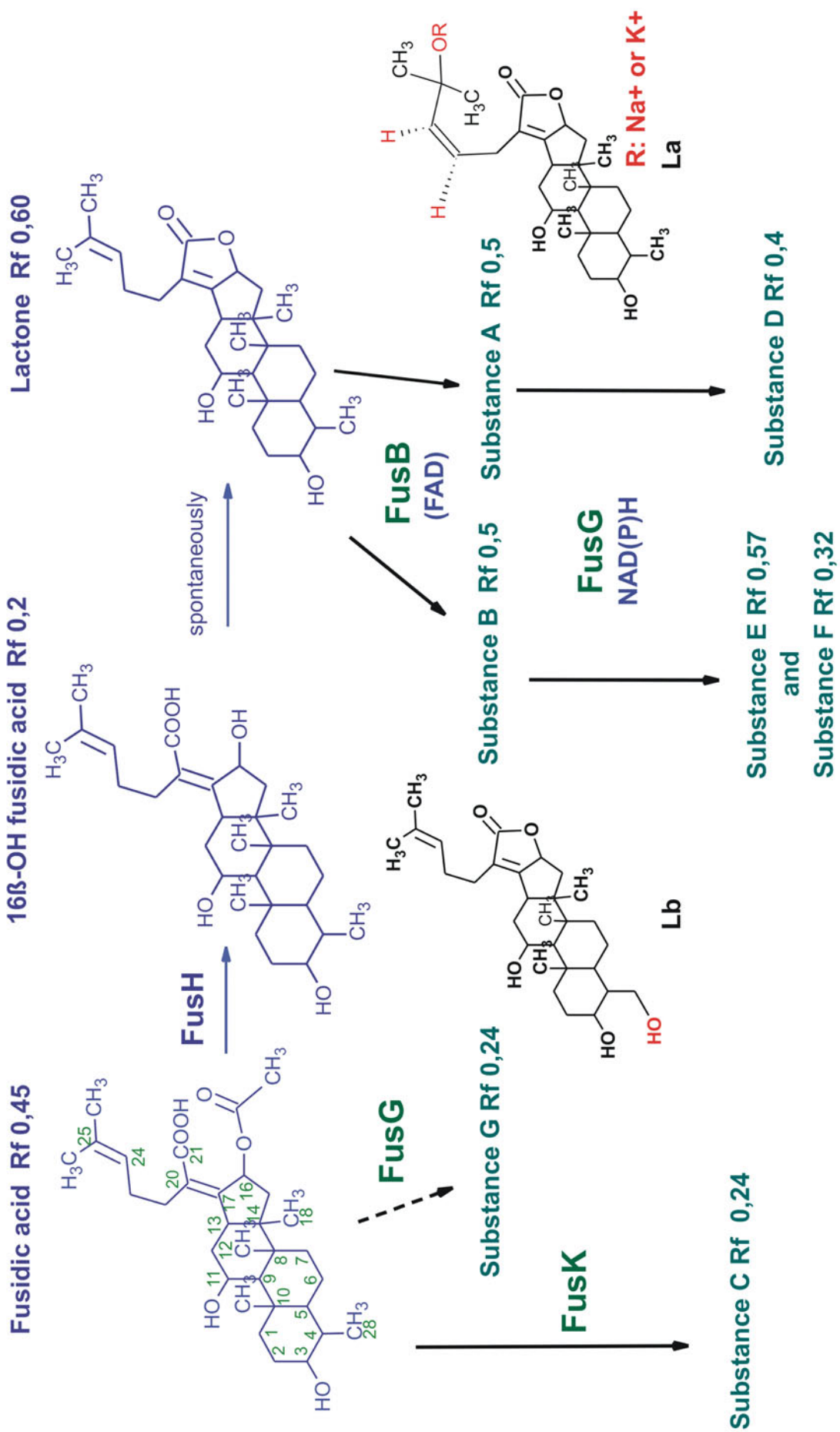
chain occupying the identical conformational space (von Daehne et al. 1979, Duvold et al. 2001).

Until now only the previous studies in our laboratory and those presented here have led to the identification and characterization of set of enzymes converting fusidic acid and its derivatives. Having optimized growth conditions, *S. lividans* was found to be a rich source of these enzymes. In addition to the newly characterized enzymes (FusB, FusG), it will be in the future interesting to deepen the knowledge on the partially characterized enzymes (FusA and FusK) as well as some of the accessory proteins. Furthermore the availability of FusB will help to gain the substrates which are converted by FusG. Another important question is the activation of the metabolites by the proposed acyl-CoA synthetase. The preliminary studies suggest that fusidic acid plays a key role in inducing the pathway for the sequential modifications. Hence it will be important to study the regulation of the corresponding genes *fus*H, *fus*A, *fus*B, *fus*G and *fus*K.

V Summary

As shown in previous studies, *Streptomyces lividans* enzymatically inactivates fusidic acid by the specific esterase FusH giving rise to the 16ß-OH derivative which spontaneously converts to the lactone. In this work it was shown that *S. lividans* further modifies fusidic acid and both derivatives which resulted in several new related substances (A, B, C, D, E, F and G). Furthermore, the enzymes involved in this biotransformation are conceivably related to the ones implicated in ß-oxidation (see attached scheme). This is supported with the following findings:

- Identification and characterization of the 30 kDa FusG enzyme which converts the substances A and B using cofactors NAD(P)H to substances D, E and F.
- The sequence analysis of the cloned *fus*G gene disclosed FusG as a homologue of hydroxyacyl-CoA dehydrogenases that are involved in oxidation of the L-3-hydroxyacyl-CoA substrates. Moreover, most of the conserved amino acids in both of their domains, important for the catalysis (including His_{146}/Glu_{158}- FusG) and dimerization are preserved in FusG, except in the region contacting the CoA moiety of the substrate.
- The lactone is converted by partially purified FusB (in the presence of FAD) to substances A and B. The loss of activity in attempts to completely purify FusB, the need of FAD in the reaction and the structure of the substance La (obtained from *S. lividans* culture filtrate) support the idea for involvement of acyl-CoA dehydrogenase, hydratase and isomerase activities.
- The gene *fus*A, coding for a putative acyl-CoA dehydrogenase, was cloned in *E. coli* and *S. lividans*. The corresponding purified proteins did not convert either fusidic acid or the lactone. Furthermore, the protein FusA (*S. lividans*) interacts with FusK.
- The 32 kDa FusK is a novel fusidic acid modifying enzyme, which gives rise to the new substance C. Immunodetection analysis (*anti*-FusH antibodies) proved that the new enzyme is not related to FusH.
- The additionally observed fusidic acid conversion by FusG (providing the substance G) needs to be clarified.
- The biotransformation of fusidic acid in *S. lividans* is so far unique, since the characterized substances (La and Lb) have not been found in either the fusidic acid producers or the fusidic acid resistant microorganisms.


Fusidic acid Rf 0,45
16ß-OH fusidic acid Rf 0,2
Lactone Rf 0,60
FusH
spontaneously
FusG
FusB
(FAD)
FusG
NAD(P)H
FusK
Substance G Rf 0,24
Substance A Rf 0,5
Substance B Rf 0,5
Substance C Rf 0,24
Substance D Rf 0,4
Substance E Rf 0,57
and
Substance F Rf 0,32
La
Lb
R: Na+ or K+

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VII Apendix

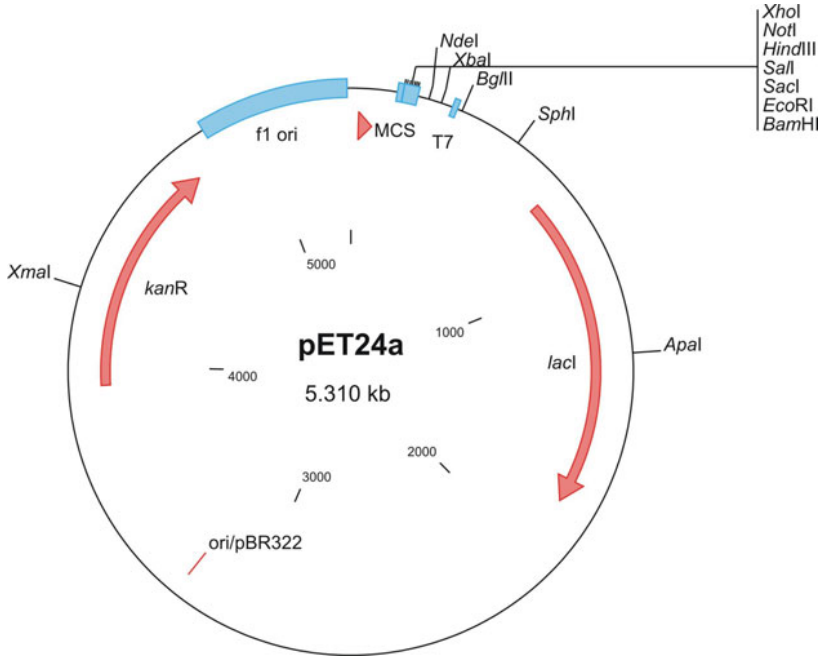
Appendix A DNA sequence of the insert of pTFG2

(The protein FusG is shown in single letter nomenclature)

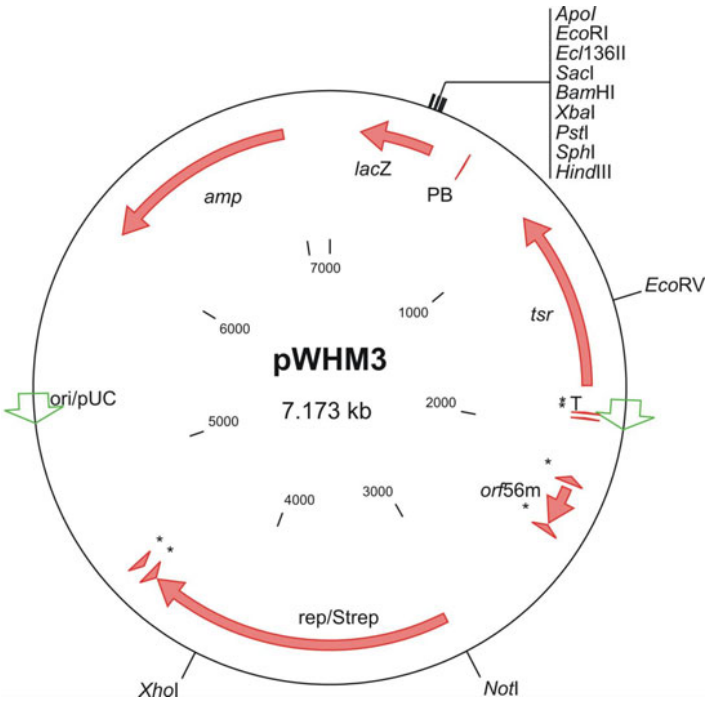
```
  1  atgcctgcga ccagccgtgg agtcagcgaa agggacgacg tgaccggcat
       M  P  A   T  S  R   G  V  S  E   R  D  D   V  T  G

 51  ctcgggagat atcgcacgtg tcggcgtggt cggctgcggt cagatggggg
     I  S  G  D   I  A  R   V  G  V   V  G  C  G   Q  M  G

101  cgggcatcgc cgaggtgtgc gcccgctcgg gtctggaggc gatggtcgcc
      A  G  I   A  E  V  C   A  R  S   G  L  E   A  M  V  A

151  gagaccaccg gcgaggctct ggagatcggc cgcacccggc tgtacaactc
       E  T  T   G  E  A   L  E  I  G   R  T  R   L  Y  N

201  gctggccaag gcggccgagc gcggcaagat gaccgaggag gagcgggacg
     S  L  A  K   A  A  E   R  G  K   M  T  E  E   E  R  D

251  cgacgcaggc gcgcctcagc ttcaccaccg acctcggcga gttcgccgac
      A  T  Q   A  R  L  S   F  T  T   D  L  G   E  F  A  D

301  cgcgatctgg tgatcgaggc cgtcgtcgag aacgagcagg tcaagaccga
       R  D  L   V  I  E   A  V  V  E   N  E  Q   V  K  T

351  gatcttccag gtgctcgatc aggtcgtgac ccggccggac gcgatcctgg
     E  I  F  Q   V  L  D   Q  V  V   T  R  P  D   A  I  L

401  cctccaacac ctcctccatc ccgctggtga agctggcggt cgccacctcg
      A  S  N   T  S  S  I   P  L  V   K  L  A   V  A  T  S

451  cggcccgacc acgtcatcgg catccacttc ttcaacccgg ccccggtgca
       R  P  D   H  V  I   G  I  H  F   F  N  P   A  P  V

501  gcagctcgtc gagctgatcc cggcgctgac cacctccgag ggcacgctca
     Q  Q  L  V   E  L  I   P  A  L   T  T  S  E   G  T  L

551  gccgggccca gctgttcacc gagaaggtgc tcggcaagca cgcgatccgc
      S  R  A   Q  L  F  T   E  K  V   L  G  K   H  A  I  R

601  gcccaggacc gctccggctt cgtggtcaac gcgctgctga tcccgtacct
       A  Q  D   R  S  G   F  V  V  N   A  L  L   I  P  Y

651  gctctccgcg atccggatgt tcgagtcggg catcgccagc cgcgaggaca
     L  L  S  A   I  R  M   F  E  S   G  I  A  S   R  E  D

701  tcgacaacgg catggagatg ggctgcgccc acccgatggg cccgctgaag
      I  D  N   G  M  E  M   G  C  A   H  P  M   G  P  L  K

751  ctggccgacc tgatcggcct ggacacggtc gcctcggtgg cgtactcgat
       L  A  D   L  I  G   L  D  T  V   A  S  V   A  Y  S

801  gtacgaggag tacaaggagc cgctgtacgc cgctcccccg ctgctccagc
     M  Y  E  E   Y  K  E   P  L  Y   A  A  P  P   L  L  Q

851  gcatggtcga cgcgggccgc ctcggccgca agagcggctc tggcttctac
      R  M  V   D  A  G  R   L  G  R   K  S  G   S  G  F  Y
```

```
901  gcctacggcc accaccacca ccaccactga c
       A  Y  G   H  H  H   H  H  H  -
```

Appendix B Restriction map of pE24a (Novagen)



Appendix C Restriction map of the shuttle vector pWHM3



Appendix D DNA sequence of the insert of pWFG1

(The protein FusG-His tag is shown in single letter nomenclature)

```
   1  gatccgtacg gtcacggatc gtgtacgggg cctcagcatg cccgacccgg
  51  gagacggaac cgtgaccatt cgggggtaac gtgctcgatc ggagcaggca
 101  ccggggaccc cggcatgcct gcgaccagcc gtggagtcag cgaaagggac
 151  gatgtgaccg gcatctcggg agatatcgca cgtgtcggcg tggtcggctg
          V  T   G  I  S   G  D  I  A   R  V  G   V  V  G

 201  cggtcagatg ggggcgggca tcgccgaggt gtgcgcccgc tcgggtctgg
      C  G  Q  M   G  A  G   I  A  E   V  C  A  R   S  G  L

 251  aggtgatggt cgccgagacc accggcgagg ctctggagat cggccgcacc
       E  V  M   V  A  E  T   T  G  E   A  L  E   I  G  R  T

 301  cggctgtaca actcgctggc caaggcggcc gagcgcggca agatgaccga
        R  L  Y   N  S  L   A  K  A  A   E  R  G   K  M  T

 351  ggaggagcgg gacgcgacgc aggcgcgcct cagcttcacc accgacctcg
      E  E  E  R   D  A  T   Q  A  R   L  S  F  T   T  D  L

 401  gcgagttcgc cgaccgcgat ctggtgatcg aggccgtcgt cgagaacgag
       G  E  F   A  D  R  D   L  V  I   E  A  V   V  E  N  E

 451  caggtcaaga ccgagatctt ccaggtgctc gatcaggtcg tgacccggcc
        Q  V  K   T  E  I   F  Q  V  L   D  Q  V   V  T  R

 501  ggacgcgatc ctggcctcca acacctcctc catcccgctg gtgaagctgg
      P  D  A  I   L  A  S   N  T  S   S  I  P  L   V  K  L

 551  cggtcgccac ctcgcggccc gaccacgtca tcggcatcca cttcttcaac
       A  V  A   T  S  R  P   D  H  V   I  G  I   H  F  F  N

 601  ccggccccgg tgcagcagct cgtcgagctg atcccggcgc tgaccacctc
        P  A  P   V  Q  Q   L  V  E  L   I  P  A   L  T  T

 651  cgagggcacg ctcagccggg cccagctgtt caccgagaag gtgctcggca
      S  E  G  T   L  S  R   A  Q  L   F  T  E  K   V  L  G

 701  agcacgcgat ccgcgcccag gaccgctccg gcttcgtggt caacgcgctg
       K  H  A   I  R  A  Q   D  R  S   G  F  V   V  N  A  L

 751  ctgatcccgt acctgctctc cgcgatccgg atgttcgagt cgggcatcgc
        L  I  P   Y  L  L   S  A  I  R   M  F  E   S  G  I

 801  cagccgcgag gacatcgaca acggcatgga gatgggctgc gcccacccga
      A  S  R  E   D  I  D   N  G  M   E  M  G  C   A  H  P

 851  tgggcccgct gaagctggcc gacctgatcg gcctggacac ggtcgcctcg
       M  G  P   L  K  L  A   D  L  I   G  L  D   T  V  A  S

 901  gtggcgtact cgatgtacga ggagtacaag gagccgctgt acgccgctcc
        V  A  Y   S  M  Y   E  E  Y  K   E  P  L   Y  A  A

 951  cccgctgctc cagcgcatgg tcgacgcggg ccgcctcggc cgcaagagcg
      P  P  L  L   Q  R  M   V  D  A   G  R  L  G   R  K  S

1001  gctctggctt ctacgcctac ggccaccacc accaccacca ctgat
       G  S  G   F  Y  A  Y   G  H  H   H  H  H   H
```

Appendix E DNA sequence of the insert of pDFA1

(The protein FusA-His tag is shown in single letter nomenclature)

```
   1  cgctgatgac ggtaggttca cgtccatggc aggcagcacc cacaccgtga
        A  D  D   G  R  F   T  S  M   A  G  S  T   H  T  V

  51  cgaatcaagc gccgccgctg accggctacg acgtcttcac ctccgaccgc
       T  N  Q   A  P  P  L   T  G  Y   D  V  F   T  S  D  R

 101  gccctggtgg aggcggtcgg gcggcagccg gcgggggagg cgcgcgagga
        A  L  V   E  A  V   G  R  Q  P   A  G  E   A  R  E

 151  ggcgctcgcc gagctgtcgg ggctcggccg gagctgcggg tcgtggcaga
       E  A  L  A   E  L  S   G  L  G   R  S  C  G   S  W  Q

 201  cccaggagtg gggagcgcag gccaacgcgc acccgccggt gctgcgcacc
       T  Q  E   W  G  A  Q   A  N  A   H  P  P   V  L  R  T

 251  cacgaccggt acggccaccg cgtcgacgag gtcgacttcc acccggcctg
        H  D  R   Y  G  H   R  V  D  E   V  D  F   H  P  A

 301  gcaccggctg ctcggcaagg gcgtctcggc gggcctgacc gacgcctggt
       W  H  R  L   L  G  K   G  V  S   A  G  L  T   D  A  W

 351  cgcggccggg cgggcacgtg cggcgcgcgg cggcgttcct gatgtggacc
       S  R  P   G  G  H  V   R  R  A   A  A  F   L  M  W  T

 401  caggtggagg cgggcaactg ctgtccgctg tcgatgaccc acgcggcggt
        Q  V  E   A  G  N   C  C  P  L   S  M  T   H  A  A

 451  cccggccctg cgcgccgacc cggaactggc cgccgagtgg gagccccggc
       V  P  A  L   R  A  D   P  E  L   A  A  E  W   E  P  R

 501  tgacctcccg ggtctacgac cgggagctgc gccccgccca tctgaaggcc
       L  T  S   R  V  Y  D   R  E  L   R  P  A   H  L  K  A

 551  ggggtgctgt tcgggatggg catgacggag aagcagggcg gcagcgacgt
        G  V  L   F  G  M   G  M  T  E   K  Q  G   G  S  D

 601  acgggcgaac agcacggcgg cgcggccgct ggccgaggac gggacgtacg
       V  R  A  N   S  T  A   A  R  P   L  A  E  D   G  T  Y

 651  agctgaccgg gcacaagtgg ttctgctcgg cgccgatgtc ggacggcttc
       E  L  T   G  H  K  W   F  C  S   A  P  M   S  D  G  F

 701  ctggtgctgg cgcgggcccc tgggggtacc gtccgtgccg aaggcaacgg
        L  V  L   A  R  A   P  G  G  T   V  R  A   E  G  N

 751  gggagggctg acctgcttcc tggtgccgcg cgtgctggcg gacggcaccc
       G  G  G  L   T  C  F   L  V  P   R  V  L  A   D  G  T

 801  gcaacgtgtt cctgatccag cggctcaagg acaagctggg caaccggtcc
       R  N  V   F  L  I  Q   R  L  K   D  K  L   G  N  R  S

 851  aacgcctccg ccgaggtcga gttcgccggg acgtgggcgc gccgggtggg
        N  A  S   A  E  V   E  F  A  G   T  W  A   R  R  V

 901  cgccgagggg cgcggggtgc ccacgatcat cgacatggtg gcggcgaccc
       G  A  E  G   R  G  V   P  T  I   I  D  M  V   A  A  T
```

```
 951  ggctggactg cgtgctgggc tcggcgggcc tgatgcgcca ggcggtggcg
       R  L  D   C  V  L  G   S  A  G   L  M  R   Q  A  V  A

1001  caggcggtgc accactgcgc gtaccgggag gcgttcggcg cgaagctggc
        Q  A  V   H  H  C   A  Y  R  E   A  F  G   A  K  L

1051  cgaccagccg ctgatgcgca acgtcctcgc ggacctggcg gtcgagtcgg
      A  D  Q  P   L  M  R   N  V  L   A  D  L  A   V  E  S

1101  aggcggccac gacgctcggg ctgcggctcg cggcggcctg cgacgccgcc
       E  A  A   T  T  L  G   L  R  L   A  A  A   C  D  A  A

1151  rccggcggcg acgagcggga gcgggcgctg ctgcggctcg cggtgccggc
        ?  G  G   D  E  R   E  R  A  L   L  R  L   A  V  P

1201  ggccaagtac tgggtgacca agcggtgcgc gccggtggtg gtggaggcgg
      A  A  K  Y   W  V  T   K  R  C   A  P  V  V   V  E  A

1251  ccgagtgcct gggcggcaac ggatacgtcg aggagtcggg cctgccccgg
       A  E  C   L  G  G  N   G  Y  V   E  E  S   G  L  P  R

1301  ctggtgcgcg agtcgccgct gaactcggtc tgggagggcg ccgggaacgt
        L  V  R   E  S  P   L  N  S  V   W  E  G   A  G  N

1351  gcaggcgctg gacgtgctgc gggcgctggg ccgggaaccg gcggcgctgg
      V  Q  A  L   D  V  L   R  A  L   G  R  E  P   A  A  L

1401  acgcgtacct gacggaggtg ggcgcggcgc gcggggccga tcaccggctg
       D  A  Y   L  T  E  V   G  A  A   R  G  A   D  H  R  L

1451  gacgcggcga tccgcgggct gctgacggag ctggccgacc tggcggccgc
        D  A  A   I  R  G   L  L  T  E   L  A  D   L  A  A

1501  cgaggggcgg gcccggctgc tggcggagcg gctggcgctg gtgctgcagg
      A  E  G  R   A  R  L   L  A  E   R  L  A  L   V  L  Q

1551  gcgcgctgct ggtgcggtac gcgccgccgg aggtcgccga cgcgttctgc
       G  A  L   L  V  R  Y   A  P  P   E  V  A   D  A  F  C

1601  gcctcgcggc tgggcggcga cgggggcgcg gcgttcggca cgctgccgcc
        A  S  R   L  G  G   D  G  G  A   A  F  G   T  L  P

1651  gaccctggac ctggcggcgg tggtggagcg ggcgcggccc gtggtccacc
      P  T  L  D   L  A  A   V  V  E   R  A  R  P   V  V  H

1701  accaccacca ccactgac
       H  H  H   H  H  -
```

Appendix F DNA sequence of the insert of pTFA1

(The protein FusA is shown in single letter nomenclature)

```
   1  tatggcaggc agcacccaca ccgtgacgaa tcaggcgccg ccgctgaccg
        M  A  G   S  T  H   T  V  T   N  Q  A  P   P  L  T

  51  gctacgacgt cttcacctcc gaccgcgccc tggtggaggc ggtcgggcgg
       G  Y  D   V  F  T  S   D  R  A   L  V  E   A  V  G  R

 101  cagccggcgg gggaggcgcg cgaggaggcg ctcgccgagc tgtcggggct
       Q  P  A   G  E  A   R  E  E  A   L  A  E   L  S  G

 151  cggccggagc tgcgggtcgt ggcagaccca ggagtgggga gcgcaggcca
      L  G  R  S   C  G  S   W  Q  T   Q  E  W  G   A  Q  A

 201  acgcgcaccc gccggtgctg cgcacccacg accggtacgg ccaccgcgtc
       N  A  H   P  P  V  L   R  T  H   D  R  Y   G  H  R  V

 251  gacgaggtcg acttccaccc ggcctggcac cggctgctcg gcaagggcgt
       D  E  V   D  F  H   P  A  W  H   R  L  L   G  K  G

 301  ctcggcgggc ctgaccgacg cctggtcgcg gccgggcggg cacgtgcggc
      V  S  A  G   L  T  D   A  W  S   R  P  G  G   H  V  R

 351  gcgcggcggc gttcctgatg tggacccagg tggaggcggg caactgctgt
       R  A  A   A  F  L  M   W  T  Q   V  E  A   G  N  C  C

 401  ccgctgtcga tgacccacgc ggcggtcccg gccctgcgcg ccgacccgga
       P  L  S   M  T  H   A  A  V  P   A  L  R   A  D  P

 451  actggccgcc gagtgggagc cccggctgac ctcccgggtc tacgaccggg
      E  L  A  A   E  W  E   P  R  L   T  S  R  V   Y  D  R

 501  agctgcgccc cgcccatctg aaggccgggg tgctgttcgg gatgggcatg
       E  L  R   P  A  H  L   K  A  G   V  L  F   G  M  G  M

 551  acggagaagc agggcggcag cgacgtacgg gcgaacagca cggcggcgcg
       T  E  K   Q  G  G   S  D  V  R   A  N  S   T  A  A

 601  gccgctggcc gaggacggga cgtacgagct gaccgggcac aagtggttct
      R  P  L  A   E  D  G   T  Y  E   L  T  G  H   K  W  F

 651  gctcggcgcc gatgtcggac ggcttcctgg tgctggcgcg ggcccctggg
       C  S  A   P  M  S  D   G  F  L   V  L  A   R  A  P  G

 701  ggtaccgtcc gtgccgaagg caacggggga gggctgactt gcttcctggt
       G  T  V   R  A  E   G  N  G  G   G  L  T   C  F  L

 751  gccgcgcgtg ctggcggacg gcacccgcaa cgtgttcctg atccagcggc
      V  P  R  V   L  A  D   G  T  R   N  V  F  L   I  Q  R

 801  tcaaggacaa gctgggcaac cggtccaacg cctccgccga ggtcgagttc
       L  K  D   K  L  G  N   R  S  N   A  S  A   E  V  E  F

 851  gccgggacgt gggcgcgccg ggtgggcgcc gaggggcgcg gggtgcccac
       A  G  T   W  A  R   R  V  G  A   E  G  R   G  V  P

 901  gatcatcgac atggtggcgg cgacccggct ggactgcgtg ctgggctcgg
      T  I  I  D   M  V  A   A  T  R   L  D  C  V   L  G  S
```

```
 951  cgggcctgat gcgccaggcg gtggcgcagg cggtgcacca ctgcgcgtac
       A  G  L   M  R  Q  A   V  A  Q   A  V  H   H  C  A  Y

1001  cgggaggcgt tcggcgcgaa gctggccgac cagccgctga tgcgcaacgt
         R  E  A   F  G  A   K  L  A  D   Q  P  L   M  R  N

1051  cctcgcggac ctggcggtcg agtcggaggc ggccacgacg ctcgggctgc
      V  L  A  D   L  A  V   E  S  E   A  A  T  T   L  G  L

1101  ggctcgcggc ggcctgcgac gccgccgccg gcggcgacga gcgggagcgg
       R  L  A   A  A  C  D   A  A  A   G  G  D   E  R  E  R

1151  gcgctgctgc ggctcgcggt gccggcggcc aagtactggg tgaccaagcg
         A  L  L   R  L  A   V  P  A  A   K  Y  W   V  T  K

1201  gtgcgcgccg gtggtggtgg aggcggccga gtgcctgggc ggcaacggat
      R  C  A  P   V  V  V   E  A  A   E  C  L  G   G  N  G

1251  acgtcgagga gtcgggcctg ccccggctgg tgcgcgagtc gccgctgaac
       Y  V  E   E  S  G  L   P  R  L   V  R  E   S  P  L  N

1301  tcggtctggg agggcgccgg gaacgtgcag gcgctggacg tgctgcgggc
         S  V  W   E  G  A   G  N  V  Q   A  L  D   V  L  R

1351  gctgggccgg gaaccggcgg cgctggacgc gtacctgacg gaggtgggcg
      A  L  G  R   E  P  A   A  L  D   A  Y  L  T   E  V  G

1401  cggcgcgcgg ggccgatcac cggctggacg cggcgatccg cgggctgctg
       A  A  R   G  A  D  H   R  L  D   A  A  I   R  G  L  L

1451  acggagctgg ccgacctggc ggccgccgag gggcgggccc ggctgctggc
         T  E  L   A  D  L   A  A  A  E   G  R  A   R  L  L

1501  ggagcggctg gcgctggtgc tgcagggcgc gctgctggtg cggtacgcgc
      A  E  R  L   A  L  V   L  Q  G   A  L  L  V   R  Y  A

1551  cgccggaggt cgccgacgcg ttctgcgcct cgcggctggg cggcgacggg
       P  P  E   V  A  D  A   F  C  A   S  R  L   G  G  D  G

1601  ggcgcggcgt tcggcacgct gccgccgacc ctggacctgg cggcggtggt
         G  A  A   F  G  T   L  P  P  T   L  D  L   A  A  V

1651  ggagcgggcg cggcccgtgg tcc
      V  E  R  A   R  P  V   V
```

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Statement

I declare that I wrote this thesis myself. I did not use other auxiliary material than indicated. Other work has been always cited.

I have not tried to get a PhD degree before.

Osnabrück, February 2004 Goran Biuković