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PhD thesis

Posttranslational modifications and structural alterations of protein synthesis elongation factor Tu in *Actinomyces* in relation to their life cycle

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Table of contents

ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
aa	amino acid
aa-tRNA	aminoacyl-tRNA
ADP	adenosine diphosphate
APS	ammonium persulfate
ATP	Adenosine 5'-Triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CE	cell crude extract
CHAPS	3-[(3-cholamiodopropyl)-dimethylammonio]-1-propanesulphonate
CoA	coenzyme A
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eEF-1A	eukaryotic translation elongation factor 1A
EF- Tu	elongation factor Tu
EF-G	elongation factor G
EF-Ts	elongation factor Ts
fMet	formyl methionine
G protein	Guanine nucleotide binding protein
GDP	Guanosine 5'-Diphosphate
GTP	Guanosine 5'-Triphosphate
IEF	isoelectric focusing
IPG	immobilised pH gradient

IPTG	isopropyl-β-D-Thiogalactopyranoside
LB	Luria-Bertani
Lit	Late Inhibitor of T4
MAC	*Mycobacterium avium* complex
MTC	*Mycobacterium tuberculosis* complex
MW	molecular weight
NEM	N-ethyl maleimide
NTM	non-tuberculous Mycobacteria
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
PMSF	phenylmethyl sulfonylfluoride
ppGpp	guanosin 5'-diphosphate 3'-diphosphate
pppGpp	guanosinpentaphosphate
P-Ser	phosphoserine
P-Thr	phosphothreonine
r.p.m	revolutions per minute
RNase	ribonuclease
S. a.	*Streptomyces aureofaciens*
S150	supernatant after sedimentation of ribosomal fraction
S30	supernatant after sedimentation of membrane fraction
Sap	spore associated protein
SDS	sodium dodecyl sulfate
TBS	Tris buffer saline
TBST	Tween containing TBS
TEMED	N,N,N',N'-tetramethylenediamine
TIR	terminal inverted repeats
Tris	tris(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolamate
X-gal	5-brom-4-chlor-3-indoyl-β-D-galactosid

1. INTRODUCTION

Protein synthesis elongation factor Tu represents one of the major components of translation system in prokaryotes. It participates on the correct positioning of the incoming aminoacyl-tRNA on the ribosome where polypeptide chain is synthesised. Besides this, EF-Tu is proposed to function in other parts of the cell metabolism. This may be the reason, why the protein is a subject of a number of posttranslational modifications such as N-acetylation, methylation and phosphorylation. In *Escherichia coli* EF-Tu located on the membrane can be methylated in the response to starvation for an essential nutrient, and it was found to be phosphorylated *in vivo* at the conserved $Threonine_{382}$. This protein functions also as an adhesion factor in *Mycoplasma pneumoniae* where it binds fibronectin and in *Lactobacillus johnsonii* where it mediates attachment of the bacteria to intestinal epithelial cells, which can play important role in virulence mechanisms of the bacteria. In addition, EF-Tu might act as a transcriptional activator in the presence of an appropriate sigma factor. Because of those important findings, EF-Tu has been an interesting object for studies by geneticists, biochemists, molecular biologists, physiologists, as well as structural biologists.

In this thesis we are comparing *Actinomyces* EF-Tu from two *Streptomyces* species with that of *Mycobacterium smegmatis*. They represent differentiating and non-differentiating *Actinomyces* species respectively and their EF-Tu primary structure is very similar. The genus *Streptomyces* is among bacteria unique since it is similar to eukaryote fungi. *Streptomyces* mycelium has also complicated life cycle with morphological and physiological differentiation, which requires communication of cells. Phosphorylation of proteins can play an important role in these communications.

The aims of this work were at first to purify EF-Tu from the wild type *Streptomyces aureofaciens* and compare it with that of tetracycline producing mutant *S. aureofaciens* 84/25, and then to look at the significance of posttranslational modifications, especially phosphorylations, of this protein during differentiation in *Streptomyces* and compare it with that of non-differentiating *Mycobacterium smegmatis*. For this purpose we used the two-dimensional gel electrophoresis and other biochemical techniques. This approach allowed us to visualize diverse heterogeneities of the protein during bacterial life cycle.

2. LITERATURE REVIEW

2.1. Elongation factor Tu

2.1.1. G-proteins

EF-Tu belongs to G-proteins. These are guanine nucleotide binding proteins that regulate a huge variety of cellular processes according to a basic reaction scheme (Bourne *et al.* 1991). When they bind GTP, they represent "on" conformation. In this state they can bind to another macromolecule or complex and trigger a distinct reaction. After a G protein has done its job, the intrinsic GTPase centre hydrolyses the GTP (in case of EF-Tu in cooperation with ribosome), and the resulting GDP induces an "off" conformation that leads to the dissociation of the G protein from the macromolecule or complex (Nierhaus 1996).

2.1.2. EF-Tu, still many questions

EF-Tu was isolated for the first time in 1964 in complex with EF-Ts, which is contrary to EF-Tu thermo stable protein (Lucas-Lenard 1971). Our significant knowledge of the protein 3D structures and its complexes obtained recently (Heffron and Jurnak 2000; Song *et al.* 1999) has allowed us to understand the structural background of most of its partial activities. Although the existing models only represent "snapshots" of the complex dynamics of the functional cycle of EF-Tu, they still facilitate studies directed to analyse the transitional changes taking place during the various interactions. Conflicting views on the function of EF-Tu in elongation, especially its energy consumption (Ehrenberg *et al.* 1990), (Rodnina and Wintermeyer 1995), still remain to be resolved. Other long known aspects such as the reason for the existence of multiple *tuf* or *tuf* like genes and their regulation require further examination. The ability of EF-Tu to function as a chaperonin (Kudlicki *et al.* 1997) and adhesin (Granato *et al.* 2004) may open more questions about other functions of this protein beside that in the protein synthesis machinery. The physiological significance of many posttranslational modifications awaits further clarification (Krab and Parmeggiani 2002). The unexplained transport of EF-Tu to the periplasm due to osmotic shock (Berrier *et al.* 2000) could also put more questions on this intricate protein. Almost all of the knowledge so far obtained about this protein comes from

studies on *E. coli*, another significant piece of knowledge was obtained from *Thermus thermophilus* and *Thermus aquaticus*. There are also attempts to explore this protein in Gram-positive bacteria, such as Bacilli (Krasny *et al.* 1998), Streptomycetes (Cappellano *et al.* 1997; Mikulik and Zhulanova 1995; Weiser *et al.* 1989) and many other microorganisms, which might illustrate more of the diversity of EF-Tu homologues although the similarity of their nucleotide and amino acid sequences are rather high. Also because of the above reason, if not stated otherwise all of the information presented in this literature review comes from the studies on *E. coli* EF-Tu.

2.1.3. Genetics

In *E. coli*, EF-Tu is encoded by two unlinked genes: *tuf*A in the *str* region and *tuf*B in the *rif* region which are located in operons containing genes of other components of protein synthesis machinery (Jaskunas *et al.* 1975), (Bosch *et al.* 1983). Their sequences are almost identical except for 13 bases, primarily in the wobble position of the codons (eleven placements). Only two out of thirteen replacements were found at the first position of the codons. One causes the only different amino acid at the C-termini of the products: Gly in EF-TuA and Ser in EF-TuB, which does not cause any obvious functional difference (Krab and Parmeggiani 1998). The second is for the initiation codon, GUG for *tuf*A and AUG for *tuf*B. Both proteins interact similarly with EF-Ts, Phe-tRNA and GDP. The rate of promoting the binding of aa-tRNA to the ribosomes is also not different.

In Gram-negative bacteria, this *tuf* gene duplication is rather common while in Gram-positive bacteria, except some Clostridia and Streptomycetes (see chapter 2.1.15.), EF-Tu is often encoded by only one gene.

2.1.4. Regulation of *tuf* gene expression

It has been speculated that two *tuf* genes may be required to produce a very high level of EF-Tu needed during rapid growth. The rate of synthesis of elongation factors is proportional to the growth rate under balanced growth conditions, and it is subject to a stringent control under amino acid starvation. *Salmonella typhimurium* strains with either of the *tuf* genes insertionally inactivated have 65 % of the wild-type EF-Tu level and those, with only one active *tuf* gene, have reduced growth and translation elongation rates (Tubulekas and Hughes 1993). Van der Meide *et al.* further found that the expression of the

two genes appeared to be coordinately regulated, maintaining an EF-TuA:EF-TuB ratio of 1.3 during different growth phases (van der Meide *et al.* 1983). A detailed mechanism of the regulation is not known but *tuf*B expression seems to be under autogenous expression control, while *tuf*A expression seems to be independent on the intracellular concentration of EF-Tu. Abdulkarim and Hughes showed that there is going on an exchange of genetic information between *tuf* genes of *Salmonella typhimurium*. In the wild type strain, this type of recombination occurs at a rate that is two or three orders of magnitude greater than the nucleotide substitution rate. This recombination phenomenon was expected to account for the concerted evolution of the *tuf* genes (Abdulkarim and Hughes 1996). Either *tuf* gene of *Salmonella typhimurium* (Hughes 1990) or *E. coli* (Zuurmond *et al.* 1999) can be deleted without loss of viability. *Tuf* gene duplication might therefore serve to secure cell viability in case that one of the genes is severely mutated. *E. coli tuf*A expression seems not to be under growth rate control, while that of *tuf*B is under growth rate and stringent control. The *in vivo* role and regulation of expression of *tuf* genes remains to be determined.

2.1.5. High conservation of the functionally important motifs in sequences through out prokaryotes

When amino acid sequences of EF-Tu (EF-1α) from many organisms are compared, it is seen that they are surprisingly well conserved through evolution. The sequences tend to be a little longer for the higher organisms due to insertions of loops in the structure (Nyborg 1998). Alignment analysis of *tuf*1-encoded protein sequences throughout the prokaryotic kingdom reveals a very high conservation in the motifs important for the interaction of EF-Tu with its ligands. As a consequence, EF-Tu phylogeny has been used to root the universal tree of life (Baldauf *et al.* 1996). As seen in Table 1, elongation factors of *Streptomyces* have higher amino acid sequence similarity with EF-Tu of Mycobacteria than with that of *E. coli* or other bacteria.

bacterial strain	*S. aureofaciens*	*S. coelicolor*	*S. collinus*	*M. smegmatis*	*E. coli*
S. aureofaciens	-	-	-	-	-
S. coelicolor	95	-	-	-	-
S. collinus	95	98	-	-	-
M. smegmatis	83	83	84	-	-
E. coli	75	75	75	75	-

Table 1. Amino acid sequence identities between the EF-Tus of three *Streptomyces* strains, *Mycobacterium smegmatis* and *E. coli*.

2.1.6. Primary structure

EF-Tu is a monomeric polypeptide, which has a molecular weight of 42-46 kDa. It is rather acidic with pI ranging from 5 to 6 (Miller and Weissbach 1970), (Blumenthal *et al.* 1972). Complete primary structure of *E. coli* EF-Tu, comprised of 393 amino acids, has been determined in 1980. The amino acid analysis of EF-Tu reveals three cysteine residues (Miller and Weissbach 1970). Two of these –SH groups are essential for the activity of EF-Tu in promoting peptide chain elongation. One of these two is required for the interaction with aa-tRNA, and readily inactivated when EF-Tu.GDP or EF-Tu.GTP is allowed to react with alkylating agents or mercury containing compounds. The other –SH group is essential for the binding of GDP or GTP, and is completely protected against N-ethyl maleimide (NEM) inactivation by binding of the nucleotide. The same –SH group essential for the GDP binding is also required for the EF-Tu-EF-Ts interaction, and is protected by EF-Ts against NEM inactivation. The third cysteine has not been observed in NEM-labelling experiments performed on the native protein.

The NH_2-terminal serine is acetylated. The sites of facile tryptic cleavage are at Arg_{44}, Arg_{58} and at Lys_{263}. The cysteinyl residues associated with aa-tRNA and guanosine nucleotide binding activities are residues 81 (Jonak *et al.* 1982) and 137, respectively. A later structural work showed that Cys_{81}, though important, is not essential for the binding of aa-tRNA to EF-Tu and it plays a more specific role in the GDP- than in the GTP-conformation (Anborgh *et al.* 1992). The COOH-terminal amino acid is heterogeneous since analyses of the COOH-terminal peptides isolated from different EF-Tu preparations

gave position 393 as Gly/Ser in ratios ranging from about 0.7 to 3. C′-terminus is probably not directly involved in any essential binding interactions with other proteins or complexes such as EF-Ts, tRNAs or the ribosome, since different C′-terminal fusions of this protein can support the complex functional repertoire of EF-Tu, but the growth rate is decreased (Schnell *et al.* 2003). On the other hand, N′-terminally tagged EF-Tu molecules were proved to be defective in function, suggesting the possible role of the N′-terminal domain in the interactions mentioned above. However, three amino acid residues long extension in the amino-terminus does not interfere with EF-Tu function (Schnell *et al.* 2003).

2.1.7. Three-dimensional structures and crystallography

Free EF-Tu is found almost always in complex with GDP, for which it has a much higher affinity than for GTP. During exponential phase, about 90 % of this protein is in complex with aa-tRNA. First 3D structure was resolved in 1985 (Jurnak 1985; la Cour *et al.* 1985). It consists of three domains. Nowadays there is a wide spectrum of all the 3D models of EF-Tu complexes with all of its ligands available, except that with the ribosome (Fig. 1).

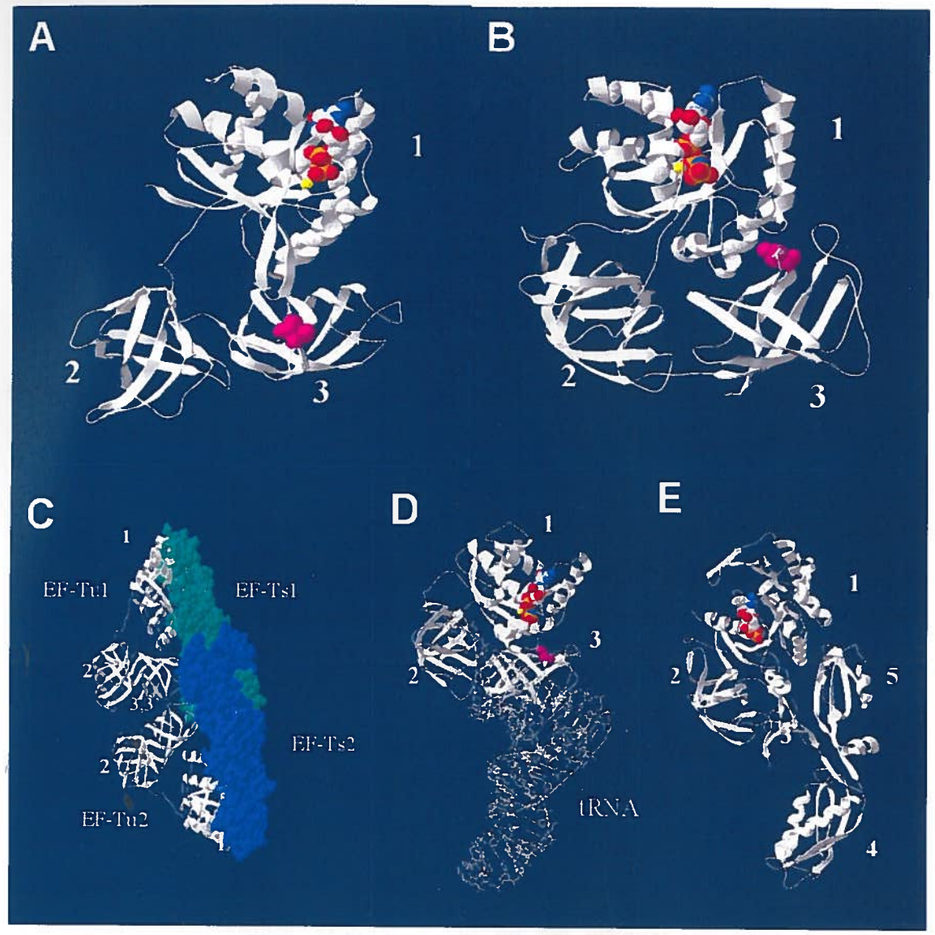


Figure 1. 3D structure of EF-Tu in complex with GDP (A), GTP (B), EF-Ts (C), and GTP.aa-tRNA (D); (E) shows the structure of EF-G.GDP mimicking the one of EF-Tu.GTP.aatRNA. Numbers designate the domains. Balls in pink color show the positions of Thr_{382} in the structures. Clusters of white, red and blue balls represent guanine nucleotides. Yellow balls represent Mg molecules. Images were made using the Swiss PDB Viewer 3.7b2 (Guex and Peitsch 1997), (http://www.expasy.ch/spdbv/mainpage.html) and sequence information was obtained from publicly available sequence databases.

When bovine mitochondrial elongation factor Ts is expressed in *Escherichia coli*, it forms a tightly associated complex with *E. coli* elongation factor Tu. These complexes were also crystallized (Karring *et al.* 2002).

Domain 1, also called G domain, (aa:1-199) has 6 β-strand core connected by loops and α-helices in a fold shared by all G-proteins even with low (< 20%) primary sequence identity. The nucleotide binding pocket is delimited by the G-protein consensus motifs. This domain is located close to the ribosomal stalk and is involved in extensive interactions with the region underneath the stalk. Helix D is involved in the initial binding of the ternary complex to the ribosome (Kothe *et al.* 2004). This domain is the most important for the function of the protein.

Domain 2 (aa: 209-299) and 3 (aa: 300-393) contain only β-strands and together behave as a rigid unit in the large conformational change from GDP- to GTP-bound state (Krab and Parmeggiani 1998). They were shown to have "selective functions" for the whole molecule, particularly toward the interaction with EF-Ts and antibiotics, the efficient interaction of EF-Tu with ribosomes and aa-tRNA, and for maintaining the differential affinity for GTP and GDP (Cetin *et al.* 1998). These domains play a crucial role in the formation of the ternary complex and in the delivery of the aa-tRNA to the ribosome. Most of the residues in domain 2, which interact with the aa-tRNA, are highly conserved between prokaryotic and mitochondrial EF-Tu (Hunter and Spremulli 2004).

However, not in all of the cases the 3D structures fit exactly to biochemical observations. One typical example could be the negative influence of His_{118} Gly substitution on aa-tRNA binding to EF-Tu observed by Jonák (Jonak *et al.* 1994).

Removing of Mg^{2+} ions from the solution, for instance with EDTA, decreases the affinity of EF-Tu towards GDP almost 1000 times, and thus it makes possible to get the nucleotide free protein. Anyway, EF-Tu without GTP or GDP looses its activity (about 10 % per hour), but when GDP is added within few hours, it is reactivated again (Fasano *et al.* 1982). GDP ensures the *in vitro* activity for several days and association with EF-Ts for several months (Parmeggiani and Sander 1981).

2.1.8. Posttranslational modifications of EF-Tu and translation regulation

2.1.8.1. Methylation

Methylation has for a long time been considered as an irreversible posttranslational modification that possesses regulatory role in cell signalling (Kraal *et al.* 1999). *In vivo* methylation of *E. coli* EF-Tu at Lys_{56} is related to the growth phase of the cells (Van Noort

et al. 1986). Lys_{56} is monomethylated during log phase and is gradually converted to N6-dimethyllysine upon entering the stationary phase. This residue of Lys_{56} takes part in interactions with nucleotides at 5′end of tRNA. However, this Lys methylation did not show any significant effect on EF-Tu affinity towards its ligands including GDP, GTP, EF-Ts and aa-tRNA but on the GTPase activity. The attenuation of GTP hydrolysis is presumed to increase the translational accuracy by prolonging the duration time for initial selection of an incoming EF-Tu.GTP.aa-tRNA. Methylation therefore slows down protein synthesis and reduces the number of misread proteins under growth limiting conditions (Kraal *et al.* 1999). It could then be a convenient supplement to regulations controlled by ppGpp (guanosintetraphosphate) and pppGpp (guanosinpentaphosphate). ppGpp makes an EF-Tu.ppGpp complex with the protein and can thus inhibit its function (Rojas *et al.* 1984). In *E. coli*, *Bacillus subtilis* and *Bacillus licheniformis*, a part of EF-Tu population that is located on the membrane can be methylated in response to starvation for an essential nutrient (Young *et al.* 1990); (Young and Bernlohr 1991). Methylation is stopped after adding this limiting nutrient and EF-Tu is slowly demethylated. EF-Tu is probably incorporated in a membrane in such a way that it can interact with one or more receptors for nutrients. EF-Tu is the only non-ribosomal protein that is known to be methylated.

2.1.8.2. Phosphorylation

The regulation of eukaryotic translation machinery by phosphorylation is well known. However, the corresponding mechanisms in prokaryotes are poorly understood. The phosphorylation seems to activate the prokaryotic translational apparatus in a generalized but not yet identified manner (Kraal *et al.* 1999).

Protein kinases play a crucial role in the regulation of many cellular processes. They alter the functions of their target proteins by phosphorylating specific serine, threonine and tyrosine residues. Several protein kinases may phosphorylate a common substrate at multiple sites.

Ribosomal protein S1, initiation factors IF1, IF2, IF3 (Robertson and Nicholson 1992), elongation factor G and the ribosomal protein S6 (Robertson *et al.* 1994) were found to be phosphorylated in *E. coli* cells infected by bacteriophage T7 and the phosphorylation of these proteins was suggested to stimulate translation of the phage late mRNAs. Protein kinases associated with ribosomes of Streptomycetes phosphorylate ribosomal proteins in bacteriophage uninfected cells, leading to a 30% loss of ribosome activity in an *in vitro* poly(U) translation (Mikulik *et al.* 1999). 5-10 % of EF-Tu from *E. coli* and *T.*

thermophilus was found to be phosphorylated *in vivo* (Lippmann *et al.* 1993) and the phosphorylated fraction remained stable under different conditions. However, extent of phosphorylation is limited by the presence of highly active phosphatase in cell extract and rapid inactivation of the kinase (Kraal *et al.* 1999). Since the phosphorylated residue (Thr_{382}) is conserved in all known EF-Tu corresponding sequences from other species, the phosphorylation might be a common phenomenon and the phosphorylated form of EF-Tu might play a fundamental role in the physiology of all organisms (Lippmann *et al.* 1993). *In vitro*, a minor amount of EF-Tu from *E. coli* could be phosphorylated by a ribosome-associated kinase (Alexander *et al.* 1995). The phosphorylation is greatly enhanced by EF-Ts and inhibited by antibiotic kirromycin. Mutants resistant to kirromycin can be phosphorylated also in the presence of this antibiotic. On the other hand, phosphorylated EF-Tu does not bind kirromycin and has higher affinity to EF-Ts. Kirromycin competes with EF-Ts for binding to EF-Tu (Abrahams *et al.* 1991). $Threonine_{382}$ from the third domain is bound by hydrogen bond to Glu_{117}. The importance of this position was tested by mutagenesis, when threonine was replaced by serine, tyrosine, alanine or aspartate. The binding of the guanosine nucleotide was not affected, but EF-Tu was able to bind aa-tRNA and be phosphorylated only in case of Thr_{382} presence (Lippmann *et al.* 1993). The highest activity of EF-Tu kinase (Ser/Thr and Tyr protein kinase(s)) was found on 70S ribosomes. EF-Tu in ternary complex cannot be phosphorylated and phosphorylated protein is unable to bind aminoacyl-tRNA (Mikulik *et al.* 1999). Because the Thr_{382} is located at a strategic position at the 1 and 3 domain interface, in the neighbourhood of hydrogen bond (Fig. 1), intercalation of the charged phosphate group changes the interaction between both domains, which abolishes its ability to bind aa-tRNA and keeps it in „GDP like conformation“ (Alexander *et al.* 1995). These findings suggest a model of EF-Tu function in which after binding of the ternary complex to the A-site of the ribosome the EF-Tu affinity to aa-tRNA is changed by GTP hydrolysis, which opens the EF-Tu complex for the kinase. Phosphorylated EF-Tu then looses its binding to the ribosomal complex, thus facilitating EF-Tu to leave the site of translation (Alexander *et al.* 1995). Phosphorylation of EF-Tu can participate in promoting the dissociation of EF-Tu from the aa-tRNA bound in A-site due to the disruption of the domain 1-3 interface (Kraal *et al.* 1999). It has been also expected to have a role in enhancing the EF-Tu affinity for EF-Ts and thus promoting GDP dissociation from the formed EF-Tu.EF-Ts complex, or direct the protein to some other functions out of the protein synthesis machinery, such as signal transduction or as a structural protein (Norris *et al.* 1996). In the study from our lab, the phosphorylation of EF-

Tu of *Streptomyces collinus*, a kirromycin producer, was demonstrated using monoclonal antibodies against phospho amino acids not only on Thr but also on Ser residues (Mikulik and Zhulanova 1995). Phosphorylation of the factor takes place also in the aerial mycelium and in spores, but to a minor extent.

It was found that *Listeria monocytogenes* possesses serin-threonine phosphatase (stp), which dephosphorylates EF-Tu regulating thus the bacterial virulence and survival in the infected host (Archambaud *et al.* 2005).

2.1.9. Phage-induced cleavage

EF-Tu is also the target for a bacteriophage exclusion system in which the host cell actively shuts down its translation (Yu and Snyder 1994). Bacteriophage exclusion is an immune-like mechanism in which bacteria commit altruistic apoptosis-like suicide in response to a phage infection, thereby preventing propagation of the phage. One of the best-studied mechanisms is that of *E. coli* K12 strain, which excludes T4 bacterial phage through the action of a metalloprotease called Lit (Late Inhibitor of T4), a host-encoded protein induced during the T4 infection. *In vivo*, Lit after it becomes activated by the Gol region in the major head protein, cleaves EF-Tu in the domain 1 region between Gly_{59} – $Ileu_{60}$, not far from the Lys_{56} and the target for trypsin cleavage (Arg_{56}-Gly_{59}), (Yu and Snyder 1994). The cleavage causes a severe inhibition of overall translation and moreover, it specifically prevents transcription of the gene downstream of the Gol site in the same T4 operon. Only the former inhibition could be alleviated by overexpression of EF-Tu. It was shown that the Gol peptide binds specifically to domains 2 and 3 of EF-Tu, creating a unique substrate for Lit, which then cleaves the domain 1 (G-domain), (Bingham *et al.* 2000). This finding gives a basis for the proposal that EF-Tu may play a role in phage head assembly and the association of EF-Tu and the T4 coat protein marks infected cells for suicide when Lit is present (Binghamet *al.* 2000). Cleaved EF-Tu has a decreased capacity to sustain *in vitro* poly(Phe) synthesis (Georgiou *et al.* 1998). Cleavage of EF-Tu by Lit is expected to labialise the local conformation, hamper GTPase centre and weaken GTP binding of EF-Tu (Kraal *et al.* 1999).

2.1.10. Spontaneous aggregation of EF-Tu

The activity in research of *in vitro* polymerisation of *E. coli* EF-Tu at low ionic strength and slightly acidic pH was renewed (Helms and Jameson 1995; Helms *et al.* 1996), together with speculations about its earlier proposed actin-like structural role (Beck *et al.* 1978). This phenomenon was described years ago for *E. coli* and *Streptomyces* (Beck*et al.* 1978), (Weiser *et al.* 1982) and then considered by many as an artefact. New studies of polymerisation of EF-Tu with numerous reports on factor's association with the membrane led to the speculation that the large aggregates may play a structural role. EF-Tu precipitates "in the presence of calcium and vinblastine", forms 6nm-repeated filaments and binds to DNase I (Beck *et al.* 1978), (Beck 1979). Weiser *et al.* also proposed that spontaneous polymerisation of EF-Tu in *Streptomyces aureofaciens* might serve as a protective mechanism for EF-Tu present in spores or enables the protein to play a structural role (Weiser *et al.* 1981; Weiser *et al.* 1982). One should also take into account that interaction with the cytoskeleton was found for EF-1α as part of actin- and tubulin-based polymeric complexes (Condeelis 1995).

2.1.11. Relative concentration and distribution of EF-Tu in bacterial cells

EF-Tu is the most abundant bacterial protein (5-10 % of the total protein), (Bosch *et al.* 1983). Rapidly growing *E. coli* with two active genes (*tuf*A(+), *tuf*B(+)) for EF-Tu contains three times as much EF-Tu (*tuf*) mRNA as EF-G (*fus*) mRNA on a molar basis, but about seven to ten times as much EF-Tu as EF-G or ribosomes (Young and Furano 1981). The molar concentration of EF-Tu is relatively higher than those of EF-G and ribosomes in part because the translation of *tuf* mRNA is more efficient than that of the other mRNAs. Cells with only active *tuf*A gene (*tuf*B(-)) increase the concentration but not the translational efficiency of *tuf* mRNA. In such cells the concentration of *fus* mRNA is almost three times higher than that of the wild-type strain. Because the *tuf*A gene is distal to but co-transcribed with the *fus* gene as part of the four-gene *str* operon, the wild-type concentration of *tuf* mRNA in these *tuf*B(-) cells must be produced by increasing the concentration of transcript corresponding to the entire str operon. Thus transcription of the *tuf*A gene can only proceed from the *str* promoter. Extracts of the *tuf*B(-) cells contain *tuf* transcripts that correspond not only in size to the entire 4.5 kb *str* operon, but also to the size (approximately 1 kb) of a *tuf* gene. Evidence suggests that this 1 kb *tuf* transcript is derived by post-transcriptional modification from the primary *str* operon transcript and that this modification could in part

explain the high translation efficiency of *tuf* mRNA (Young and Furano 1981). EF-Tu concentration is equivalent to that of aa-tRNA so that nearly all of the cellular aa-tRNA are trapped by EF-Tu.GTP (Gouy and Grantham 1980).

In *Streptomyces*, both distinct morphological forms, the vegetative mycelium and dormant spores, contain high concentrations of EF-Tu (Weiser *et al.* 1989).

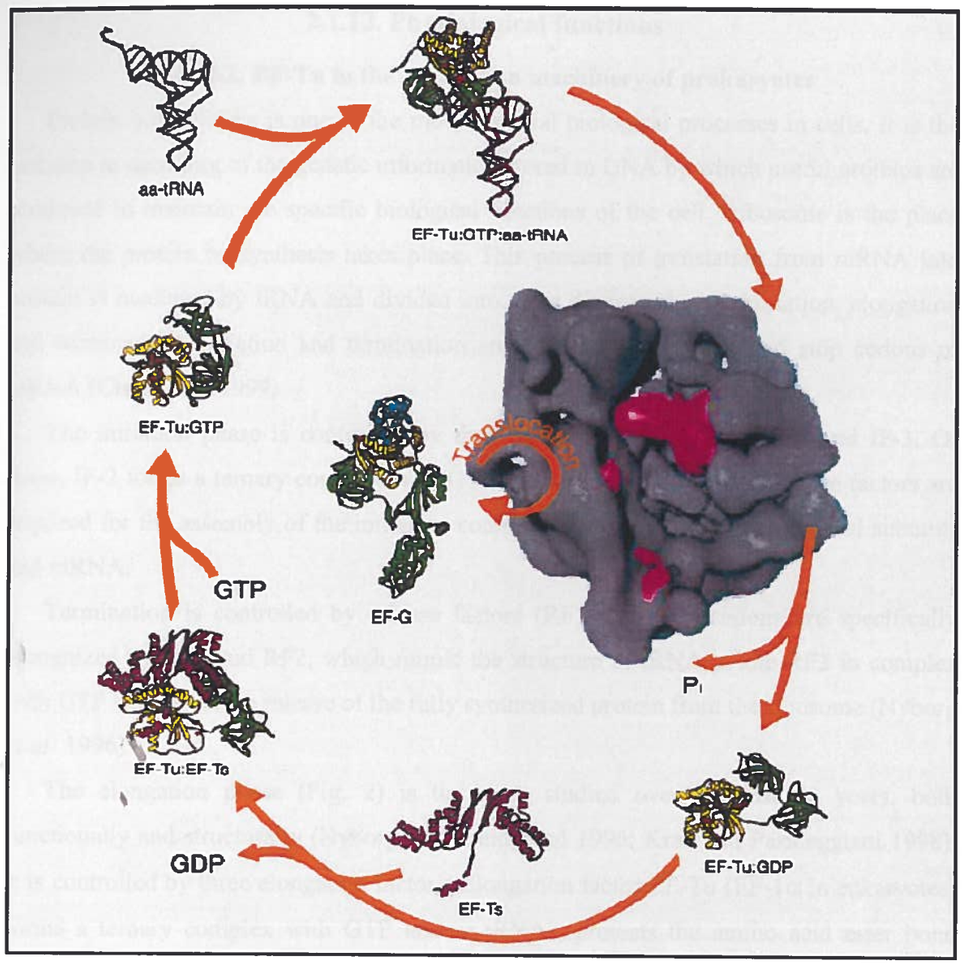


Figure 2. The functional cycle of elongation factor Tu. At the top is the ternary complex of Phe-tRNA:EF-Tu:GDPNP with Phe-tRNA. Going round clockwise is the ribosome particle (not in scale). Next is the EF-Tu:GDP after release from the ribosome. At the bottom is EF-Ts entering the complex of EF-Tu:EF-Ts for nucleotide exchange. Finally, the active EF-Tu:GDPNP is found ready for interacting with aa-tRNA. In the middle is shown EF-G:GDP, which in its active form translocates tRNAs on the ribosome. The image is taken from (Clark *et al.* 1999).

2.1.12. Physiological functions

2.1.12.1. EF-Tu in the translation machinery of prokaryotes

Protein biosynthesis is one of the most essential biological processes in cells. It is the last step in decoding of the genetic information stored in DNA by which useful proteins are produced to maintain the specific biological functions of the cell. Ribosome is the place where the protein biosynthesis takes place. This process of translation from mRNA into protein is mediated by tRNA and divided into three distinct phases: initiation, elongation and termination. Initiation and termination are directed by the start and stop codons of mRNA (Clark *et al.* 1999).

The initiation phase is controlled by three initiation factors IF-1, IF-2 and IF-3. Of these, IF-2 forms a ternary complex with GTP and initiator $tRNA_{fMet}$. All three factors are required for the assembly of the initiation complex with $tRNA_{fMet}$, the ribosomal subunits and mRNA.

Termination is controlled by release factors (RF). The stop codons are specifically recognized by RF1 and RF2, which mimic the structure of tRNA, while RF3 in complex with GTP stimulates the release of the fully synthesized protein from the ribosome (Nyborg *et al.* 1996).

The elongation phase (Fig. 2) is the most studied over the last 30 years, both functionally and structurally (Nyborg and Kjeldgaard 1996; Krab and Parmeggiani 1998). It is controlled by three elongation factors. Elongation factor EF-Tu (EF-1α in eukaryotes) forms a ternary complex with GTP and aa-tRNAs, protects the amino acid ester bond against hydrolysis and carries the aa-tRNA to the ribosomal A-site for decoding of mRNA by codon-anticodon interactions. When correct codon-anticodon recognition occurs, GTP hydrolysis on EF-Tu is stimulated by the ribosome and EF-Tu:GDP is released. Then the aa-tRNA is brought into contact with the peptidyl-tRNA in the ribosomal P-site, where peptide bond formation is catalysed adding one amino acid to the growing peptide. The nucleotide exchange factor EF-Ts (EF-1β in eukaryotes) converts EF-Tu:GDP into the active EF-Tu:GTP. The last elongation factor EF-G (EF-2 in eukaryotes) in complex with GTP controls the translocation of tRNAs and mRNA on the ribosome. Several years ago it has been postulated that there are two molecules of GTP hydrolysed with EF-Tu per one peptide bond formed, which indicates that the stoichiometry of this complex might not be 1:1:1, but rather 2:2:1 (Ehrenberg *et al.* 1990). This finding would change dramatically the view on the role of EF-Tu in elongation and on the overall energetic balance of translation

process. Accumulation of these new data and an older findings of „Leiden" and „Upsala" groups concerning co-operative effects of EF-Tu mutants led Leendert Bosh and his co-workers to a formulation of a model of revised bacterial polypeptide chain elongation cycle (Bosch *et al.* 1996). It supposes that two molecules of EF-Tu operate on the ribosome successively in one cycle, which then in stepwise manner increases the restriction of unwanted ternary complexes. The question remains whether this scheme is in accordance with kinetic and accuracy measurements performed by an „Uppsala" group showing that both GTPs are cleaved at the same time (Scoble *et al.* 1994).

2.1.12.2. EF-Tu and translation accuracy

The *in vivo* overall missense error frequency in translation was found rather low, in the range of 10^{-3}-10^{-4}, which requires more control than just simple codon-anticodon interaction. A model for translation accuracy control proposed by Hopfield and Ninio (Hopfield 1974; Ninio 1975) is composed of two main steps: the initial selection of the proper ternary complex by the mRNA-programmed ribosome, rejecting noncognate ternary complexes, and proof reading process following GTP hydrolysis, discarding near-cognate ternary complexes. By this way, the incoming ternary complex is checked twice before it is boarded onto the ribosome and a peptide bond is formed. Computer models were also used to evaluate the influence of EF-Tu concentration on the dynamics and fidelity of protein synthesis (Pingoud *et al.* 1990). The accuracy of protein synthesis is influenced by various genetic and environmental variables, which implies that these variables have different effects on the outcome of the ribosome's reaction with cognate and noncognate aa-tRNAs (Thompson 1988). It also indicates the need for signal transfer between translation machinery and the environment. The ppGpp has been reported to increase the accuracy of translation *in vivo* (Rojas *et al.* 1984). Under *in vitro* conditions, this molecule slows the rate of incorporation of amino acids into the protein through association with EF-Tu. EF-Tu.ppGpp complex can re-associate with the ribosome and prevent peptide bond formation. By showing that the rate constants characterizing the ribosome interaction with EF-Tu are independent of whether the aa-tRNA is cognate or noncognate, Thompson suggested the use of these rate constants as an internal standard to measure the translational accuracy through measuring the ribosome's interaction with the aa-tRNA (Thompson 1988). In the same manner, Parker *et al.* used EF-Tu in a high-resolution two dimensional gel electrophoresis to study mistranslation and the stringent control system in bacterial cells which is related to the ability of the bacteria to accumulate (p)ppGpp in response to amino

acid starvation (Parker and Friesen 1980; Parker *et al.* 1978). However, computer simulations of the elongation cycle of bacterial protein biosynthesis further demonstrated that the accuracy of protein biosynthesis could not be explained by a mechanism, which involves only an initial selection and a proofreading reaction. It was suggested that a combination of initial selection, proofreading and a retardation of non-cognate flows at the level of the EF-Tu-catalysed GTPase reaction and the peptidyl transfer could guarantee sufficient accuracy at reasonable costs (Pingoud *et al.* 1990). According to this view the ribosome functions as an allosteric enzyme, which in its affinity and enzymatic activity responds optimally only to the cognate substrate.

2.1.13. EF-Tu multifunctionality

Nowadays it is being more often realized that many housekeeping proteins have many other functions than previously thought. EF-Tu is not the exception. The abundance of this protein in bacterial cells, supported by the studies of EF-Tu subcellular distribution and content, has inspired researches to look for possible functions of the factor besides that in the translation system. EF-Tu has been expected to function either as a structural protein (Beck *et al.* 1978) or as a transcription regulator (Travers 1973). Recently it was found that eukaryotic translation elongation factor 1A (eEF-1A) of *Saccharomyces cerevisiae* binds actin and these binding regions do no participate in regulation of the translation elongation in yeast (Gross and Kinzy 2005). Not fully understood is also its role as one of the four subunits of the replicase of RNA phages, where EF-Tu acts in a complex with EF-Ts in the initiation step (Blumenthal *et al.* 1972). A recent novel finding about EF-Tu is the chaperon-like activity. *In vitro* assays showed that EF-Tu could act as a chaperon on the renaturation of urea-denatured rhodanese (Kudlicki *et al.* 1997), a model substrate used in protein folding studies. Nucleotide-free EF-Tu from *Thermus thermophilus* can promote partial renaturation of 1:1 stoichiometric amount of rhodanese but its action becomes much more efficient in complex with GTP and in the presence of EF-Ts, in conditions inducing multiple rounds of GTP hydrolysis. The activity depends on the reversible transitions between "on" and "off" states. It is reduced when it is "frozen" by kirromycin or pulvomycin in GDP conformation. The evidence of this activity of EF-Tu was later broadened to other denatured proteins such as citrate synthase, and α-glucosidase. EF-Tu associates with unfolded proteins and protects native proteins against heat denaturation (Caldas *et al.* 1998). It was shown that EF-Tu, like DnaK, interacts with several

hydrophobic peptides contained in the sequences of the membrane protein phospholemman, lysozyme, and alkaline phosphatase (Malki *et al.* 2002). This property is probably specific to chaperones. Krab and Parmeggiani proposed that EF-Tu might somehow contribute to the induction of the correct folding of the nascent polypeptide chain on the ribosome, with a mechanism involving a GTP hydrolysis independent from that associated with the binding of the ternary complex to the A site. This activity could also be an alternative explanation of the consumption of two molecules of EF-Tu.GTP in poly(Phe) synthesis (Krab and Parmeggiani 1998). It certainly needs more attempts to clarify these speculations and possibilities but these findings undoubtedly open promising and innovative development.

During last few years this protein was found to function as an adhesion factor. EF-Tu of *M. pneumoniae* binds fibronectin as part of a virulence mechanism (Dallo *et al.* 2002). EF-Tu also mediates attachment of *Lactobacillus johnsonii* to intestinal epithelial cells and mucins and stimulates proinflammatory reactions (Granato *et al.* 2004). Furthermore, this membrane surface conformation of EF-Tu not only confers new biological and virulence-related functions but also identifies novel vaccine candidates and targets for anti-infective therapies (Dallo *et al.* 2002).

Upon osmotic downshock, a few cytoplasmic proteins including EF-Tu and DnaK are released to the periplasm of *E. coli*, mostly via the mechanosensitive channel MscL (the channel with the largest pore). Blocking of this channel impairs the release (Berrier *et al.* 2000).

2.1.13.1. EF-Tu as a signal transmitter

In *E. coli*, translation and transcription of stable RNA is a subject not only to stringent control linked to amino acid starvation, but also to a more general growth rate control. The nucleotide ppGpp has been implicated in the mechanism through which both controls operate (Doull and Vining 1995). EF-Tu was postulated to interact with one or more nutrient sensing proteins. Young and Bernlohn proposed that the nutrient-dependent methylation of EF-Tu might be involved in the regulation of growth, possibly as a principal component of an unidentified signal transduction pathway in bacteria (Young *et al.* 1990); (Young and Bernlohr 1991). The proposal has been well supported by a number of observations: (i) EF-Tu promotes rRNA synthesis which is inhibited by ppGpp (Travers 1973); (ii) it is associated with the periplasmatic space and outer membrane (Young and Bernlohr 1991); (iii) it can be transported from the cytoplasm to the periplasm of *E. coli*

during osmotic downshock presumably via a mechanosensitive channel (Berrier *et al.* 2000); (iv) it is very abundant and interacts with GDP and GTP whose stock pool affect bacterial differentiation (Itoh *et al.* 1996; Ochi 1986) and (v) the expression of some *tuf* genes is under stringent-control, for instance in *M. xanthus*, where EF-Tu expression was recovered during development (Horiuchi *et al.* 2002).

The stringent response is an important signal for the entry into the developmental process, and *M. xanthus relA* mutant has been shown to be unable to develop. The regulation of *Streptomyces* secondary metabolism (Guthrie and Chater 1990), cellular differentiation (Kwak *et al.* 1996; Leskiw *et al.* 1991), carbon catabolism and cell-cell signalling (Kataoka *et al.* 1999) has become more manifested recently. This tRNA acts as a regulator by recognizing rare codons present only in certain pathway-specific genes and hence switching on their expression. Clearly, tRNA does not interact with the ribosome alone but is presented in the form of a ternary complex with EF-Tu and GTP. The molecular mechanism of tRNA selection on ribosome has however remained elusive. Based on biochemical and genetic evidence, Powers and Noller suggested a model in which EF-Tu upon interaction with ribosome accepts signalling from the 530 loop of 16 rRNA concerning proper selection of incoming aa-tRNA (Powers and Noller 1994).

2.1.14. EF-Tu inhibitors

EF-Tu is known as the target of four groups of antibiotics, with kirromycin, pulvomycin, antibiotic GE2270A, and Enacyloxin IIa as representatives.

Kirromycin reduces the affinity of EF-Tu.GTP towards the aa-tRNA (Abrahams *et al.* 1991) but the ternary complex can still bind to the ribosome. However, after the hydrolysis of GTP, antibiotic blocks the release of EF-Tu.GDP from the ribosome. This blocking activity inhibits further steps of peptide bond formation and immobilizes the ribosomal complex on the mRNA, thus causing a "traffic jam" for the whole polysome queuing on the same mRNA. In addition, various evidences indicate that the allosteric control mechanism is highly perturbed by kirromycin (Abrahams *et al.* 1991). It alters the behaviour of EF-Tu by mimicking its natural effectors in: (i) stimulating GDP release (like EF-Ts), (ii) stabilization of GTP binding (like aa-tRNA), (iii) stimulation of GTPase centre (like ribosomes), (iv) enzymatic binding of aa-tRNA in the absence of GTP, (v) inhibiting the binding of EF-Ts. In the presence of salts, binding of kirromycin has a stabilizing effect on EF-Tu (Sedlak *et al.* 2002).

Pulvomycin does the matter by preventing the binding of aa-tRNA to EF-Tu even in the presence of GTP and the suggested inhibition mechanism was that of substrate limitation (Kraal *et al.* 1995). Like kirromycin, all the parameters of the interaction with GTP and GDP are changed, indicating that also pulvomycin perturbs the allosteric control mechanism of EF-Tu. Nevertheless, the binding site for pulvomycin is different from that of kirromycin (Zuurmond *et al.* 1999). Pulvomycin resistant mutants showed to be mutated in the three-domain interface of EF-Tu.GTP (Zeef *et al.* 1994), which is also crucial for the GTPase switch movement. Pulvomycin affects both the GDP- and GTP-bound conformations of EF-Tu, enhancing both dissociation and association rate of EF-Tu.GDP (Anborgh and Parmeggiani 1993). The reason for the blocking effect still remains unclear.

Antibiotic GE2270A acts on EF-Tu in a similar mode as pulvomycin by preventing the formation of a stable complex between EF-Tu.GTP and aa-tRNA (Anborgh and Parmeggiani 1991). GE2270A binds to both EF-Tu.GDP and EF-Tu.GTP but it only interferes with the kinetics of GTP dissociation, thereby stabilizing an inactive GTP-bound conformation of EF-Tu (Anborgh and Parmeggiani 1993).

Enacyloxin IIa has been found to inhibit protein synthesis also by binding to EF-Tu (Watanabe *et al.* 1992), (Cetin *et al.* 1996). In the presence of Enacyloxin IIa, EF-Tu.GTP can form a stable, but anomalous complex with aa-tRNA. Consequently, aa-tRNA is mispositioned in the A site and the peptidyl transferase reaction is blocked. However, *in vitro* experiments showed that the antibiotic might directly act on the ribosome A site without binding with EF-Tu. Enacyloxin IIa also increases GDP/GTP exchange rate and inhibits EF-Ts binding but it does not enhance the intrinsic GTPase activity of EF-Tu as does kirromycin.

2.1.15. EF-Tu in *Streptomyces*

In *Streptomyces*, beside the *tuf*1 gene, which encodes for constitutive EF-Tu, there are one or two other *tuf*-like genes, which rather differ from *tuf*1. *S. ramocissimus tuf*2 and *tuf*3 show 89 % and 63 % identity to *tuf*1, respectively. *S. collinus, S. lividans* and *S. coelicolor* do not have *tuf*2-like gene (Krab and Parmeggiani 1998). The *tuf*3 transcription of *S. coelicolor* A3(2) occurring after nutritional shiftdown (which resulted in disappearance of *tuf*1 transcripts) was followed by the production of ppGpp, which indicates that *tuf*3 is a subject of positive stringent control (van Wezel *et al.* 1995).

Streptomyces cinnamoneus EF-Tu is naturally kirromycin resistant (Cappellano *et al.* 1997). Interestingly, when the Thr_{378} was mutated to the consensus Ala, the resulting mutant protein was sensitive to kirromycin but retained some activity (30 % of the control) even at high kirromycin concentrations (Cappellano *et al.* 1997).

EF-Tu1 from *Streptomyces collinus*, another kirromycin producer that has Ala_{378} naturally occurring in its sequence, is kirromycin sensitive and so is the EF-Tu1 from *S. coelicolor*. Using two-dimensional electrophoresis and immunodetection with antibodies against P-Ser and P-Thr, it was found that *S. collinus* factor is phosphorylated on threonine and serine. The level of phosphorylated EF-Tu was claimed to vary during growth and differentiation (Mikulik and Zhulanova 1995).

The relative content of EF-Tu and ribosomes found in dormant spores of *Streptomyces aureofaciens* was very similar to that found in exponentially growing vegetative cells (Weiser *et al.* 1989). Cell-free homogenates from spores were found to contain EF-Tu cleaved by membrane-bound protease. The protease cleaved purified aggregated EF-Tu much less efficiently than non-aggregated factor in cell homogenates.

The *in vivo* and *in vitro* transcription analysis revealed two transcriptional start sites for *tuf*1 gene in the kirromycin producer *Streptomyces ramocissimus*. Transcription from these two promoters appeared to be growth-phase dependent, diminishing drastically upon entry into the stationary phase and at the onset of kirromycin production. In surface-grown cultures (spores on agar plates), a second round of *tuf*1 transcription, coinciding with aerial mycelium formation and kirromycin production, was observed. During exponential growth, the transcripts from both promoters are present at comparable levels. In contrast to *E. coli*, which has two almost identical *tuf* genes, *S. ramocissimus* contains only *tuf*1 coding for its regular EF-Tu. High levels of EF-Tu may therefore be achieved by the compensatory activity of the extra promoter for *tuf*1 (Tieleman *et al.* 1997).

2.1.16. EF-Tu in *Streptomyces aureofaciens*

The filamentous soil bacterium *Streptomyces aureofaciens* is a producer of tetracyclines. These antibiotics act on ribosomes, where they compete for binding with ternary complexes. Tetracycline production strains possess many mechanisms for resistance against this antibiotic. Product of the gene *Tet*M, which has N-terminal high sequence homology with EF-Tu could act as a protein preventing the binding of tetracycline on ribosome or tetracycline resistant analog EF-Tu (Sanchez-Pescador *et al.*

1988). Therefore it is interesting to study, beside for other reasons, the characteristics of EF-Tu from this strain.

In *Streptomyces aureofaciens* both distinct morphological forms, the vegetative mycelium and dormant spores, contain high concentrations of EF-Tu (Weiser *et al.* 1989). Weiser described spontaneous polymerisation of EF-Tu from *Streptomyces aureofaciens*, which might serve as a protective mechanism for EF-Tu present in spores or enables the protein to play a structural role (Weiser *et al.* 1982).

Our sequence data show that one of the phosphorylation sites on *S. aureofaciens* EF-Tu is very probably Thr_{386} analogous to Thr_{382} in *E. coli* in the middle of conserved sequence at the C-terminal end of the protein. This phosphorylation probably plays a principal role in elongation cycle and the phosphate group is added and removed from the protein with a rate corresponding to the rate of protein synthesis *in vivo* (in *E. coli* over 10 amino acids per ribosome per second). This implies that both protein kinase and phosphatase doing the job might be localized on the ribosome (see chapter 2.1.8.2.).

2.2. *Streptomyces*

Streptomyces are Gram-positive bacteria belonging to the *Actinomycetales*. Their most interesting property is the ability to produce a multitude of varied and complex secondary metabolites, some of which have an important role in a medicine and veterinary practice. Their most common role is to be used as antibiotics, but they have also many other useful biological properties such as modulation of the immune system and acting as enzyme inhibitors.

In contrast to other bacteria, *Streptomyces* undergo a sophisticated developmental cycle in which only the spore stage is unigenomic. This includes differentiation into at least three distinct cell types (Hodgson 2000).

2.2.1. The ecological niche of Streptomycetes

Streptomyces inhabit mostly soil and water ecosystems where their role appears to be as general saprophytes. They excrete extra-cellular enzymes hydrolysing polymers (remains of other organisms) such as starch or cellulose, and absorb the soluble breakdown products (Hodgson 2000). This makes *Streptomyces* central organisms in carbon recycling

(Bentley *et al.* 2002). The soil environment is therefore carbohydrate-rich but relatively nitrogen and phosphate-poor. On the other hand, such oligotrophic environment also shelters a very large number of other microbes including bacteria, fungi and protists, which could be serious competitors for *Streptomyces*. Even though there is a great deal of nutrient input, the size of soil organism's population is so large that it is very rapidly exhausted. While in a laboratory under rich nutrient conditions Streptomycetes can have generation times as short as one hour, in woodland soil the generation time for Streptomycetes was estimated as 1.7 days (Hodgson 2000).

2.2.2. Cultivation conditions in laboratory

In their dynamic natural environments, Streptomycetes are usually exposed to both, solid phase (for instance the surface of soil particles) and liquid phase (water flows beneath soil particles or currents of raining water) bringing many stimuli from surroundings simultaneously (Chater 2001). In contrast, the laboratory liquid cultures are usually fixed in one developmental phase. The biggest advantage of laboratory culture conditions is that it allows microbiologists to have a pure culture on which studies could be done. Another advantage is that fixed conditions cause fewer variables affecting bacterial physiology (or is it a disadvantage?). In the laboratory, besides being cultivated in liquid media with shaking, like other bacteria, *Streptomyces* are cultivated in semisolid media, which contain agar as a solidifying agent. Optimal conditions for *Streptomyces* growth are 28-30 °C temperature and aeration. The *Streptomyces* colonies growing on a solid surface usually consist of different cell types and it is almost impossible to obtain completely homogenous cell population from a given differentiation phase to study their physiology and metabolism.

Liquid culture is usually considered to contain exclusively one cell type as substrate mycelium. However, there are numerous reports that some *Streptomyces* can produce spores in liquid culture and others can go through a microsporulation cycle (Koepsel and Ensign 1984), (Stastna *et al.* 1977). In liquid culture, some *Streptomyces* have the tendency to form pellets and to grow on the walls of the flask above the tidemark, especially in minimal media (Hodgson 2000). The cells inside the pellet are physiologically different from those outside. Some *Streptomyces* produce many secondary metabolites at high levels only when they are in their natural habitat.

Because the liquid or solid agar medium cultivation systems are rather far from the situation in nature and do not allow simple and reproducible preparation of cell homogenate protein samples from different stages of *Streptomyces* mycelium growth, there was therefore developed a new two-phase cultivation system for *Streptomyces* in our laboratory (Nguyen *et al.* 2005). This system is composed of a solid phase formed by tiny glass beads (Balotina) surrounded by a complex liquid medium (Fig. 3). The glass beads, originally used as a reflective additive to the street paints, function as a matrix resembling soil or sand particles and give mechanical support for cell growth and differentiation. The liquid medium serves as a dynamic phase which allows easy replacement of nutrients and growth factors as well as labelling of cell proteins *in vivo* (during differentiation), (Nguyen *et al.* 2005), (Kofronova *et al.* 2002). Our previous experiments also showed that there are more pigments (antibiotics) produced by *Streptomyces* when cultivated on glass beads than when they are grown in liquid medium or on the agar plates. Furthermore, due to the original industrial utilization of Balotina, this technique is quite cheap.

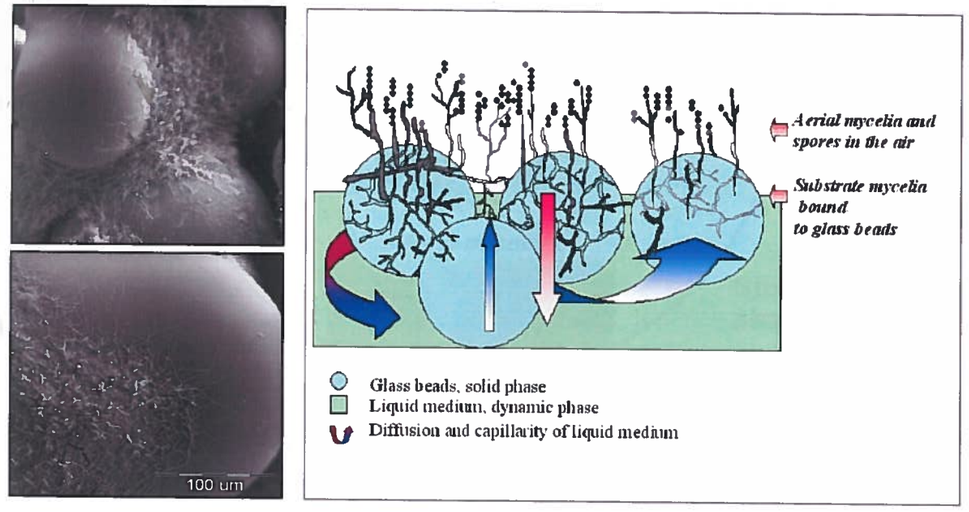


Figure 3. The glass beads cultivation system. Scheme and an image from scanning electron microscope of *Streptomyces* colony growing on glass beads.

2.2.3. Morphological differentiation

2.2.3.1. Germination

A typical *Streptomyces* life cycle is summarized in Fig. 4, starting from germination of spores. Germination in a liquid culture is characterized by three distinct stages: the change from a bright phase to a dark phase appearance in the microscope, swelling of the spores and outgrowth of the germ tube (Hardisson *et al.* 1978). Germination in all Streptomycetes can be initiated in complex media, which include amino acids, vitamins and nucleotides. However, in defined media the requirements are species specific, such as a mild heat shock.

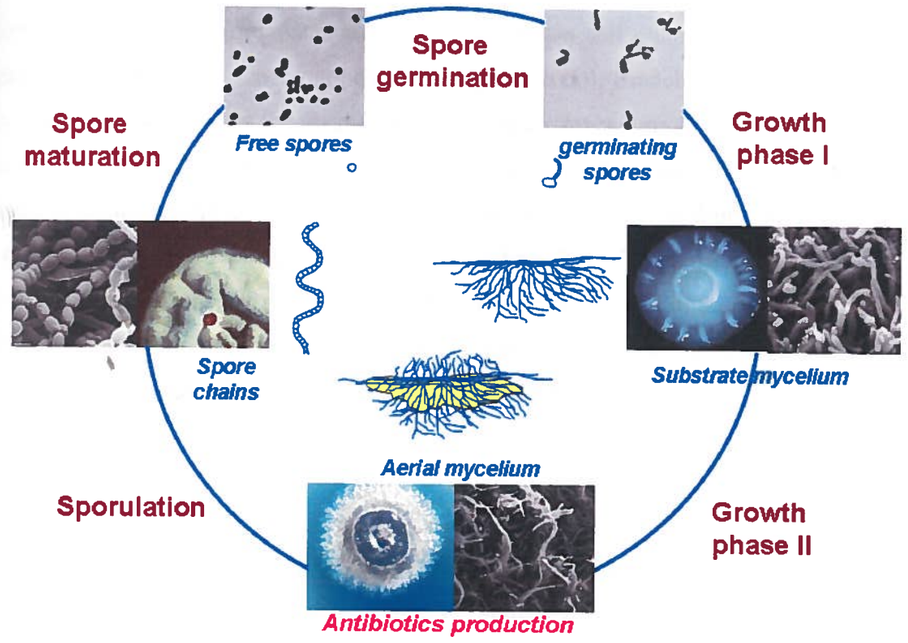


Figure 4. Schematic presentation of morphological and biochemical differentiation of *Streptomyces* during their life cycle.

The first stage of germination requires divalent cations such as Ca^{2+}, Mg^{2+}, and Fe^{2+}. In minimal media, this stage strictly requires CO_2, which is incorporated into oxalacetate, presumably by pyruvate carboxylase (Grund and Ensign 1978). This implies the stimulating role of CO_2 on the Krebs cycle. Swelling of *S. antibioticus* spores leads to a 1.5 fold increase of their volume without any concomitant increase in weight. Outgrowth of *Streptomyces* involves DNA, RNA and protein synthesis, during which exogenous carbon

and nitrogen sources are required. There is a concomitant increase in cell mass, but a reduction of respiratory quotient to that of vegetative cells (Hodgson 2000). The primary germ tube, which immerses from a spore, is followed by the emergence of a second germ tube from the same spore later.

2.2.3.2. Substrate mycelium

One of the problems of a saprophytic life cycle, in which insoluble polymers are digested and the resulting hydrolysate is taken up and used in cells, is that this process takes time and relies on a high concentration of the digestive enzymes. Some microbes solve this problem by the substrate mycelium. The advantage of hyphal cells is that they can cross relatively large distance within and between soil crumbs to reach for nutrients present in soil only in separated patches (Hodgson 2000). Branching of primary filaments or germ tubes leads to the formation of substrate mycelium of the young colony. *S. coelicolor* A3(2) hyphae, as other *Streptomyces*, grow by apical extension. Unseptated hyphae contain many copies of the chromosome. On minimal medium the colony at first consists of radially growing major hyphae, then branches arise at intervals along the major hyphae and begin to colonize the areas between them, some turning to grow in a radial direction and thus increasing the number of major hyphae as the circumference of the colony growth. The hyphae may vary in diameter but their appearance is almost identical (Hopwood 1960).

2.2.3.3. Aerial mycelium

After a short pause in the growth, called the transition phase (Granozzi *et al.* 1990), (Novotna *et al.* 2003), caused by nutrient limitation, aerial mycelium rises from the substrate mycelium out in the air (Kelemen and Buttner 1998).

Before that the colony starts to accumulate storage materials, for instance glycogen, which creates sufficient osmotic pressure inside of the cell and helps to erect the hyphae (Chater 1989). The first aerial hyphae, having hydrophobic outer layer, protrude upwards from the radially growing major hyphae of the substrate mycelium, and others develop later from the smaller side-branches. To support aerial hyphae growth and subsequent development, some parts of the substrate mycelia undergo an orderly process of internal dismantling including extensive chromosome digestion leading to a programmed cell death (Miguelez *et al.* 1999; Wildermuth 1970). Aerial mycelium also uses storage compounds for growth from the substrate mycelium, before it lyses.

The formation of aerial hyphae in *Streptomyces* has been defined genetically by isolation of *bld* mutants that do not form aerial mycelium and therefore have a shiny, 'bald' appearance (Chater 2001), (see further).

2.2.3.4. Septation and sporulation

Streptomyces and many other Actinomycetes have developed the ability to form hydrophobic exospores or arthrospores. Sporulation is initiated by coiling of aerial hyphae, which are then divided into spore-sized compartments by special cross-walls, the "sporulation septa" differing from the vegetative ones by the presence of two membranes separated by two layers of the cell wall (Wildermuth and Hopwood 1970), (Wildermuth 1970). This process is accompanied by an orderly segregation of the population of chromosomes into compartments each containing a single genome. There are 5 to 50 spores (often pigmented) in one chain. When the septum is completed, glycogen is degraded and its carbon skeleton is incorporated into the walls and spores mature. Once a colony is washed with water the hydrophobic spores are spread due to their surface tension.

The biological role of Streptomycetes spores is most probably as dispersal agents rather than resting stages (Hodgson 2000). The induction of sporulation is maintained by transcription sigma factors, which modify the specificity of RNA polymerase turning on the expression of differentiation genes (Chater 1984).

2.2.4. Physiological differentiation

Physiological differentiation in *Streptomyces* normally refers to the production of secondary metabolites. Temporal association of production of secondary metabolites, especially antibiotics, and morphological differentiation is a long known phenomenon. The genes for antibiotic production are activated during a lag ("hesitation") phase of the growth (Holt *et al.* 1992) and are always clustered (Martin 1992). In the cluster there is often present a gene for membrane protein, which is likely to be responsible for the export of antibiotic out of the cell, and one or more genes for antibiotic resistance. Among these resistance genes, at least one is clustered with the structural genes or sometimes the resistant gene is one of the structural genes of the biosynthesis pathway (Davies 1992). Interestingly, antibiotic producers are not always necessarily resistant to their own products added exogenously to the medium before they start their production (Li 1993). In many

cases, there is a regulatory gene for antibiotic production also clustered with the structural genes.

First, the basic skeleton of the antibiotic is synthesized, which is then modified. Actinorhodin for instance is synthesized by polyketidic row from malonyl CoA (Hopwood and Sherman 1990).

At least one antibiotic, paramycin, was found to promote morphological differentiation (Kondo *et al.* 1988) but the principle function of antibiotics is more likely in ecological competition, by acting against other organisms than the producer. A plausible argument is that antibiotics can serve to defend the lysing colony against invasion of motile organisms during aerial mycelium formation (Chater 1989).

Streptomyces produce 75 % of commercialised antibiotics from which the most important commonly used are e.g. streptomycin (producer *S. griseus*), tetracycline (producer *S. rimosus*) or chloramphenicol (producer *S. venezuelae*). One *Streptomyces* species might produce many structurally unrelated antibiotics.

In addition to antibiotics, the secondary metabolites include siderophores (e.g. coelichelin), pigments (e.g. tetrahydroxynaphtalen), lipids (e.g. hopanoids), polyphosphate and other molecules such as butyrolactones or geosmin responsible for typical soil odour of *Streptomyces* (Bentley *et al.* 2002).

2.2.5. Genome of *Streptomyces*

Regulation of differentiation in *Streptomyces* is proved to be a multilevel and complex process, which is reflected by almost the largest genome (8.6 Mbp) among the bacteria (Hodgson 2000). To compare, *E. coli* has 4.6 Mbp and a simple eukaryote – the yeast *Sacharomyces cerevisiae* has 13 Mbp genome size. *Streptomyces* genetic information is carried on a linear chromosome with terminal inverted repeats (TIRs) with covalently bound protein molecules on the free 5′ ends, and on some linear as well as circular plasmids.

Nearly all the essential genes and the *oriC* are located in the core (e.g. in the centre) of the chromosome while the non-essential genes lie in the arms. The sequenced genome of *Streptomyces coelicolor* (8,667,507 base pair) is estimated to encode 7,825 theoretical proteins and contains an unprecedented proportion of regulatory genes (for example a remarkable 65 sigma factors of which 45 are extra-cytoplasmic function sigma factors). They are predominantly involved in responses to external stimuli and stresses. There are

many duplicated gene sets that may represent 'tissue-specific' isoforms operating in different phases of colonial development, which is a unique situation for a bacterium (Bentley *et al.* 2002). The genome analysis also revealed abundant two-component regulatory systems. Approximately 35 % of the *S. coelicolor* genome is annotated as encoding 'hypothetical' proteins of unknown function, and 182 proteins of this type were identified on the proteome map (Hesketh *et al.* 2002).

Plasmids, ranging from 9 kb to 350 kb, carry genes that confer advantageous phenotypes, e.g. those responsible for formation of antibiotics, degradation of xenobiotics and heavy-metal resistance (Meinhardt *et al.* 1997).

Both the chromosomal and plasmid DNA have high G+C content (70 – 74 %), and both contain principally similar structural features, namely terminal inverted repeats (TIR) carrying covalently bound protein molecules on the free 5′ends.

2.2.6. Regulation of *Streptomyces* differentiation

As was mentioned earlier, morphological differentiation and antibiotic production are under overlapping or identical control. From the genetic point of view, from studies of mutants, the best characterized stages are the formation of aerial mycelium and sporulation. Nearly all of the *bld* mutants of *Streptomyces coelicolor* that lack the ability to form aerial mycelium (Chater 2001) can be made to produce aerial mycelium either by changing the medium composition (especially by replacing glucose in minimal medium with manitol) or by growing them near the wild-type strain (Chater 1993).

Mutants unable to produce spores are called *whi* (white), because they stay white instead of developing the normal grey surface colour conferred by a spore-wall associated polyketide pigment (Chater 2001). Nine *whi* loci (*whi*A, *whi*B, *whi*C, *whi*G, *whi*H, *whi*I, *whi*J, *whi*D, *whi*E) have been so far analysed (Chater 1993), most of them coding proteins with regulatory function (Chater 2001). The *whi*G gene encodes sigma factor that is involved in the beginning of sporulating cascade. Other loci are responsible for the synthesis of spore associated polyketide pigment (*whi*E), (Kelemen and Buttner 1998) or in the spore wall synthesis (*whi*D), (Chater 1993). While *whi* mutations appear only to affect the differentiation of aerial hyphae into spores, *bld* mutations have pleiotropic effects that generally prevent antibiotic production. They also cause defects in carbon catabolite repression or in cell to cell signalling leading to blocking of differentiation (Kelemen and Buttner 1998).

Other mutants prepared in *Streptomyces coelicolor* and *Streptomyces lividans* (*afs*, *abs*) are morphologically normal but fail to produce antibiotics. One of the *afs* genes – the *afsR* is a pleiotropic effector and essential regulatory gene for the biosynthesis of an A-factor (A factor is an γ-butyrolacton, see later) and the antibiotics actinorhodin and undecylprodigiosin (Hong *et al.* 1991). The induction of actinorhodin production by *afsR* was found to occur through the transcriptional stimulation of antibiotic biosynthetic genes (Hong *et al.* 1991). *Abs* mutants (antibiotic synthesis deficient) of *Streptomyces coelicolor* failed to produce any of the four antibiotics normally synthesized by the wild type strain. There were identified two *abs* loci: *absA* encoding two-component signal transduction system and *absB* coding RNAase III.

2.2.6.1. Regulation by a rare codon tRNA

A range of circumstantial evidence suggests that genes required for vegetative growth in *Streptomyces* do not contain the leucine codon TTA. Instead, the codon seems to be confined to a few genes necessary during differentiation, when the colonies begin to produce aerial hyphae and antibiotics (Lawlor *et al.* 1987; Leskiw *et al.* 1991). Mutations in *bld*A, the structural gene for Leu-tRNA$_{TTA}$, do not retard vegetative growth, but they prevent normal aerial mycelium formation and antibiotic production (White and Bibb 1997). TTA codons present in some genes, mostly genes for morphological differentiation and antibiotic production, make their expression dependent on *bldA* (Leskiw *et al.* 1991) and the processed BldA product is available only during later state of the growth (Leskiw *et al.* 1993). However, *bld*A gene, in addition to its well-documented affects on stationary-phase processes, is important also for the correct functioning of at least some processes that do not persist into the stationary phase (Kim *et al.* 2005).

Transcription of *adp*A, a regulatory gene needed for colonial morphogenesis in *Streptomyces coelicolor*, is activated during aerial hyphae formation and its TTA triplet is required for aerial mycelium formation (Nguyen *et al.* 2003). Developmental genes containing the rare TTA codon in *Streptomyces* may be preferentially translated during starvation responses associated with antibiotic biosynthesis and colonial morphogenesis, since under amino acid starvation conditions charged tRNA isoacceptors corresponding to preferred codons are probably depleted first and those corresponding to rare codons remain relatively abundant.

adpA$_c$ is a *S. coelicolor* gene similar to the *Streptomyces griseus* A factor-regulated *adpA*$_g$. Both of these genes contain a TTA codon. A TTA-free version of *adpA*$_c$, obtained by replacing the TTA (leucine) codon by a cognate TTG (leucine) could partially restore aerial mycelium formation. This indicates that the UUA codon in *adpA*$_c$ mRNA is principal target through which *bldA* influences morphological differentiation (Takano *et al.* 2003).

The TTA-containing regulatory gene SCO4263 of *S. coelicolor* is associated with the absence of few proteins from the membrane proteome of bldA mutant and appears to regulate a cluster of operons of function-unknown genes (Kim *et al.* 2005).

2.2.6.2. Posttranslational modifications

An average of 1.2 proteins per gene was observed in *S. coelicolor* proteomic studies, indicating extensive posttranslational regulation (Hesketh *et al.* 2002). While signal-transduction pathways differ in their complexity, they function mainly through protein phosphorylation catalysed by protein kinases in both prokaryotes and eukaryotes. This leads to changes in gene expression or enzymatic activity that enable cells to generate an appropriate response to a given signal (Zhang 1996). Eukaryotic-type protein kinase inhibitors were found to inhibit *in vitro* phosphorylation of some proteins and morphological differentiation of *Streptomyces griseus* (Hong *et al.* 1993). An *in vitro* phosphorylation reaction with crude cellular extracts also revealed that phosphorylation of a 17-kDa protein probably on a tyrosine residue *is* associated with the onset of *S. griseus* aerial mycelium formation on solid medium. It is proposed that this 17-kDa protein may play a role in cellular differentiation of *S. griseus* via its phosphorylation (Okamoto *et al.* 1998). During germination, phosphorylation/dephosphorylation of proteins participates on developmental processes (Mikulik *et al.* 2002). Serine/threonine kinases in *Streptomyces granaticolor* (Pkg2, Pkg3 and Pkg4) play also some role in a developmental process (Vomastek *et al.* 1998). An active form of Pkg4 is able to autophosphorylate itself at threonine residue(s). Protein kinase Pkg2, which has broad substrate specificity, is the first transmembrane protein serine/threonine kinase described for Streptomycetes and could serve as a receptor for developmental signals (Nadvornik *et al.* 1999). This kinase could probably target number of proteins and so modulate their functions, in which case it might be assumed that Pkg2 occupies a high position in a hierarchy of signalling cascade.

The *bld*B and *bld*D genes of *S. coelicolor* A3(2) encode putative transcription factors. Their sequencing and analysis revealed unique tyrosine residues essential for BldB and BldD function, raising the possibility that these tyrosine residues might be potential targets for phosphorylation that regulates the activities of the factors. In *S. griseus,* phosphorylation of a 17 kDa protein, probably also on a tyrosine residue, has been shown to occur at the onset of aerial mycelium formation (Okamoto *et al.* 1998), and the 18 kDa calculated molecular weight of *S. coelicolor* BldD is close enough to indicate the possibility that these two proteins might have same function (Kelemen and Buttner 1998). Recently, sequence analysis showed that these proteins are both BldD and their amino acid sequence identity is 98.2 % (http://www.expasy.org).

The pattern of ADP-ribosylated proteins in *S. coelicolor* also changes during morphological differentiation. Several *bld* mutants *(bld*B, *bld*C and *bld*H) are defective in ADP-ribosylation and a new "bald" locus, *brg*A, has been identified by mutations conferring resistance to 3-aminobenzamide, an inhibitor of ADP-ribosyltransferase (Shima *et al.* 1996). Changes in ADP-ribosylation are also associated with morphological differentiation in *Myxococcus xanthus*, although their clear-cut regulatory role in bacterial differentiation has not yet been shown (Kelemen and Buttner 1998).

2.2.6.3. Signalling pathways

The influence of carbon source on morphological differentiation indicates that the carbon metabolism and morphological differentiation are interlinked in *Streptomyces*. The possibility of extracellular complementation of the mutants suggests that there might be involved some extracellular signalling in the regulation of morphological differentiation. The signalling is very often mediated by small diffusible molecules.

There is an increasing evidence for the involvement of a complex extracellular signalling cascade in the programmed differentiation of *S. coelicolor* growing on rich media (Nodwell *et al.* 1996; Willey *et al.* 1993). When pairs of *bld* mutants are grown on R2YE in close proximity, one of the mutants can induce the other to erect aerial hyphae and consequently sporulate. This "extracellular complementation" is always unidirectional, with one *bld* mutant acting as a "donor" and the other as a "recipient" (Kelemen and Buttner 1998). Complementary experiments with available *bld* mutants revealed the following putative cascade, in which each mutant can rescue the developmental defect in all the mutants to the left, but not to the right:

Direction of complementation

[bld261] < *[bld*K*]* < *[bld*A, *bld*H*]* < *[bld*G*]* < *[bld*C*]* < *[bld*D*]*.

Direction of the cascade

In this proposed cascade, each extracellular signal produced by a mutant induces the synthesis and/or release of the next signal, eventually causing the *bld*D-dependent production of SapB (spore associated protein, which is present also in aerial mycelium), and perhaps other morphogens, which allow aerial hyphae to overcome surface tension of the environment and grow into the air (Fig. 5), (Willey *et al.* 1993; Nodwell *et al.* 1996). At present, there is a biochemical evidence only for "signal 1" and sapB (shown in Fig. 5), (Nodwell and Losick 1998). Exogenous addition of purified SapB only leads to aerial hyphae formation but not to sporulation as does extracellular complementation (Tillotson *et al.* 1998). This implies that SapB functions only as a surfactant to decrease the surface tension at the colony surface and thus allows the erection of aerial hyphae. Other gene products are required for complete morphological differentiation in bacteria. The proposed signalling cascade could, however, serve for the integration of various environmental and metabolic signals leading to the bacterial decision to differentiate. These signals might be expected to include nutritional status (Merrick 1976; Champness 1988) and cell density (Nodwell *et al.* 1996). In this context, it is interesting to note that most of the *bld* mutants are defective in the regulation of carbon utilization (Pope *et al.* 1996). Their inability to differentiate was proposed to be a secondary consequence of their inability to sense and/or signal starvation. This is also in agreement with the fact that their morphological defects are conditional on carbon source, because wild-type strains of *S. coelicolor* undergo complete morphological differentiation without the production of SapB, indicating that alternative pathways for aerial hyphae emergence exist. So far there have been identified 12 *bld* loci most of them coding regulatory proteins (Chater 2001).

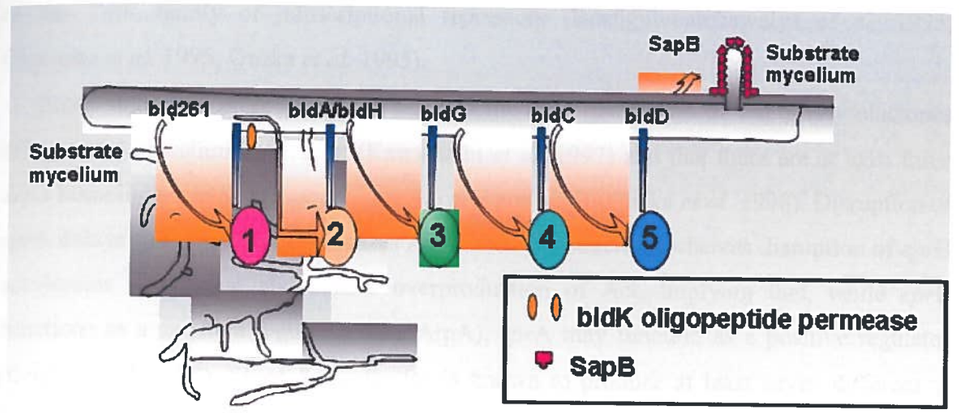


Figure 5. A model for a regulatory signal cascade in *S. coelicolor*. The figure represents the growth of substrate mycelium on a rich medium such as R2YE. Signal 1 (Nodwell and Losick 1998) is produced in a bld261-dependent manner and accumulated extracellularly. It is taken up by the BldK oligopeptide permease, triggering synthesis and release of signal 2, and so on, leading to the eventual BldD-dependent production of SapB and potentially other morphogens, which lead to the production of aerial mycelium. The figure was redrawn after (Kelemen and Buttner 1998).

2.2.6.4. Pheromones

Several *Streptomyces* species control their morphological differentiation and secondary metabolism by pheromones. These are low-molecular compounds that are released from the cells and are assumed not to play functional role inside of the producers, but they may work as a defense weapons against other organisms or as autoregulators of cellular differentiation in *Streptomyces* (Beppu 1995). γ-butyrolactones have long been known to play an important role in controlling both secondary metabolism and morphological differentiation in several *Streptomyces* species (Horinouchi and Beppu 1992). The most studied γ-butyrolactone, A-factor, is essential for both streptomycin biosynthesis and aerial mycelium formation in *S. griseus*. These compounds are structurally similar to homoserine lactone molecules involved in quorum sensing in Gram-negative bacteria, but it is not yet clear whether γ-butyrolactones function as signals for starvation, cell density, or some other parameters in Streptomycetes (Neumann *et al.* 1996). γ-butyrolactones act via receptor proteins (ArpA in the case of A-factor) with DNA-binding domains that are related to those

of the TetR family of transcriptional repressors (Ruengjitchatchawalya *et al.* 1995; Okamoto *et al.* 1995; Onaka *et al.* 1995).

From studies in *S. coelicolor* it has become clear that some of the γ-butyrolactones affect aerial mycelium formation (Kawabuchi *et al.* 1997) and that there are at least three *arpA* homologues in *S. coelicolor* - *cpr*A, *cpr*B and *scb*R (Onaka *et al.* 1998). Disruption of *cpr*A delays sporulation and decreases Act and Red production, whereas disruption of *cpr*B accelerates sporulation and causes overproduction of Act, implying that, while *cpr*B functions as a negative regulator (like ArpA), *cpr*A may function as a positive regulator (Onaka *et al.* 1998). Since *S. coelicolor* is known to produce at least seven different γ-butyrolactones (Kawabuchi *et al.* 1997), important question for the future will be to determine whether each receptor responds to a different γ-butyrolactone, or whether all are recognized by one receptor, and how their DNA-binding activities respond to cognate factor(s).

2.2.6.5. Intracellular metabolic signals

In *E. coli*, the highly phosphorylated nucleotide ppGpp functions as an intracellular signal for starvation. It is synthesized by spoT and RelA, ribosome bound enzymes that are activated by uncharged tRNAs binding to the activator site of translating ribosomes. In *S. coelicolor*, under conditions of nitrogen limitation, *rel*A null mutants not only fail to produce Act or Red antibiotics, but also show a marked delay in the onset and extent of morphological differentiation (Chakraburtty and Bibb 1997). These results imply that ppGpp plays an important role in the induction of differentiation under conditions of nitrogen limitation.

In *M. xanthus* ppGpp, synthesized in response to amino acid limitation, induces developmental gene expression leading to multicellular fruiting body formation, and a mutation in *rel*A arrests the development prior to aggregation (Harris *et al.* 1998). In addition, increasing of the intracellular concentration of ppGpp by artificially expressing *E. coli rel*A is sufficient to initiate developmental gene expression (Singer and Kaiser 1995).

In *S. coelicolor* expressing *rel*A under the control of an inducible promoter causes precocious aerial mycelium formation and sporulation under conditions of nutritional sufficiency (Kelemen and Buttner 1998). The same effect was observed in *sbl*A mutants of *S. lividans*. Glucose-grown cultures of this *S. lividans* mutant enter earlier into stationary phase than glucose-grown wild-type cultures. This mutant strain expresses *aml* gene

encoding α-amylase, normally strongly glucose-repressed gene, even in the presence of glucose (Gagnat *et al.* 1999).

It is noticeable that the *tuf*3 gene in *Streptomyces coelicolor* A3(2) is a subject to stringent control induced by ppGpp (van Wezel *et al.* 1995). Ochi observed a significant intracellular accumulation of ppGpp and the coordinated decrease in the GTP pool that occurs at the onset of morphological differentiation (Ochi 1987; Ochi 1986). Since ppGpp is synthesized from GTP, and IMP dehydrogenase (an enzyme required for GMP, and hence GTP synthesis) is inhibited by ppGpp, ppGpp synthesis and a drop in the GTP pool normally occur simultaneously. This makes it hard to distinguish which might be acting as a trigger. However, GMP synthetase inhibitor decoyinine causes a decrease in the GTP pool without inducing ppGpp synthesis. Because decoyinine induces aerial mycelium formation at concentrations that only partially inhibit growth, Ochi proposed that morphological differentiation is a direct consequence of the decrease in the GTP pool and not of the ppGpp synthesis (Ochi 1987; Ochi 1986). Moreover, a gene *(obg*) encoding a GTP-binding protein with GTPase activity suppresses aerial hyphae formation in *S. griseus* when introduced on a multicopy plasmid (Okamoto *et al.* 1997). This might suggest that Obg might sense intracellular GTP concentration as an indicator of the physiological state of the hyphae and in response initiate morphological changes. In this context, EF-Tu could also do the same and trigger a special regulation in translation machinery adaptive to the new bacterial physiological state.

2.2.6.6. Role of translation in the regulation of *Streptomyces* developmental processes

Studies investigating the relationship of osmotic shock with development in *Streptomyces coelicolor* led to the identification of at least four SigB-like sigma factors (SigH, SigI, SigJ, and SigX), from which the transcription of *sig*H gene is controlled by stress regulatory systems and the developmental program in *S. coelicolor*. Posttranslational control mechanisms eliminate the SigH N-terminal extension during later stages of growth and development (at the time of aerial mycelium formation) and SigH-$s_{51/52}$ is processed into 34 kDa (and probably 38 kDa) products via cleavage at sites near the SigH-s_{37} translation initiation site (Viollier *et al.* 2003). Processing is mediated by a protease found in later stage liquid cultures. SigH forms truncated products, which appearance might reflect translational or posttranslational events. The fact that both SigH-$s_{51/52}$ and SigH-s_{37} each have their own translational start sites, and that accumulation of SigH-s_{37} is coordinated with decreases in SigH-$s_{51/52}$, might be explained by a hypothetical

translational switching mechanism coordinated with protease activities that cleave SigH-$s_{51/52}$ into SigH-s_{38} and, subsequently, SigH-s_{34}. Proteolytic cleavage is a targeted processing event presumably generating a SigH-s_{37}-like product likely to have altered activity. These studies of SigH isoforms revealed the activity of processing enzymes that may serve as general regulators of developmental genes.

Another case of posttranslational modification connected with cell differentiation is *bld*N, which is one of a genes required for the formation of specialized, spore-bearing aerial hyphae during differentiation in *Streptomyces coelicolor*. This gene encodes a member of the extracytoplasmic function subfamily of RNA polymerase sigma factors. The primary translation product is a proprotein (pro-sigma(BldN)) that is proteolytically processed to a mature species (sigma(BldN)) by removal of the unusual N-terminal extension, which is concomitant with aerial mycelium formation (Bibb and Buttner 2003).

2.3. Mycobacteria

Mycobacteria belong also to the order *Actinomycetales*, so they are taxonomically related to Streptomycetes. Many Mycobacteria are harmless and useful because they degrade organic matter in soil. More attention, however, attract the pathogenic Mycobacteria, which cause among others the ancient diseases tuberculosis or leprosy. Mycobacteria are unicellular, aerobic bacteria, considered to be Gram-positive, with a G+C rich genome (61-71 %). They are rather pleomorphic, they may vary in form from coccobacilli to long slender rod-shaped cells and they may even undergo branching and filamentous growth, but true mycelium is not formed.

The Mycobacteria are unusual among bacteria in that they have an enormously thick hydrophobic cell wall that endows them with resistance to dehydration, acids and alkali. The cell wall is composed of long-chain fatty acids, called mycolic acids, complex waxes and unique glycolipids. Other important wall components are trehalose dimycolate (so-called cord factor) and Mycobacterial sulfolipids, which may play a role in virulence. Another unique constituent, which may contribute to pathogenesis, is lipoarabinomannan (LAM). The lipid contents of the cell wall may be as high as 60 % of its dry weight. This unique cell wall provides an initial barrier to antibiotics and other chemotherapeutic agents.

It is responsible for the hydrophobic nature of Mycobacteria, which allows them to float on the surface of aqueous media, and also for their distinctive staining property.

2.3.1. Classification of Mycobacteria

With the exception of *Mycobacterium leprae*, the etiologic agent of leprosy, the Mycobacteria are classified into two broad categories: members of the *Mycobacterium tuberculosis* complex causing the tuberculosis and nontuberculous Mycobacteria (virtually all other species), which are ubiquitous in the environment, mostly non pathogenic, only some of them are opportunistic pathogens. Additionally, Mycobacteria are most frequently divided, according to their growth rate, into slowly (e.g. *M. tuberculosis*, *M. leprae*) and rapidly growing species (e.g. *M. smegmatis*, *Mycobacterium abscessus*). The most rapidly growing species grow on simple media after incubation for 2 - 3 days at temperatures between 20 - 40 °C; however, the slow growers as the pathogens from the MTC have the generation time of 18-24 hours and require incubation for 2 - 6 weeks or more on enriched media. Of the pathogenic species, *M. leprae* has never been cultivated successfully *in vitro*, so it appears to be an obligate intracellular pathogen that requires the environment of the host macrophage for survival and propagation. Estimates of its generation time *in vivo* are in the order of 10 to 12 days.

2.3.2. *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex (MTC) comprises the species *M. tuberculosis, M. bovis, M. microtii, M. africanum* and *M. canetti*, which all can cause tuberculosis in humans or animals. Although tuberculosis has been a preventable and curable disease for over 40 years, *Mycobacterium tuberculosis* is still an enormous source of human suffering (Dye *et al.* 2002). It is estimated that tuberculosis caused 30 million deaths worldwide in the last decade, making it the leading cause of death associated with infectious disease. Tuberculous Mycobacteria enter the alveoli by airborne transmission. They resist destruction by alveolar macrophages and multiply, forming the primary lesion or tubercle; then they spread to regional lymph nodes, enter the circulation, and reseed the lungs. Tissue destruction results from cell-mediated hypersensitivity.

2.3.3. Non-tuberculous Mycobacteria

Non-tuberculous Mycobacteria (NTM) are saprophytic organisms living freely in soil and water, some of them are opportunistic pathogens as those belonging to the *Mycobacterium avium* complex (MAC). Non-tuberculous Mycobacteria are classified by pigmentation in the light or dark and by growth rate. NTM were not widely recognized as human pathogens until the 1950s. Numbers of potential pathogens and prevalence have increased over the past two decades, largely in association with the AIDS epidemic (Falkinham 1996). The infection caused by NTM is not transmissible between humans, but it is acquired from natural sources (e.g. soil and water).

2.3.4. Mycobacteria genomics and proteomics

A model system for proteomics and structural genomics of Mycobacteria is *M. tuberculosis*. Together with the promise of structures of proteins with novel folds, high-resolution structures of drug targets are providing the basis for rational inhibitor design, with the goal of the development of novel anti-tuberculars and an effective vaccine against this persistent pathogen (Smith and Sacchettini 2003).

Recently, a microarray assay based on DNA gyrase B subunit (gyrB) gene sequences was developed (Fukushima *et al.* 2003). This can be used for the rapid identification of Mycobacteria species isolates, which is necessary for the effective control of tuberculosis. Direct comparison of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* genomes revealed presence of an additional gyrB in *M. smegmatis* flanked by novel genes. Analysis of the amino acid sequence of gyrB from different organisms suggests that the orphan gyrB in *M. smegmatis* may have an important cellular role (Jain and Nagaraja 2002).

The proteome of an organism or cell reflects its functional status in response to physiological and environmental conditions. Proteomics can be used to complement genomic investigations and provides the opportunity to determine which ORFs of a genome are actually translated into functional proteins. Furthermore, it may be used to examine the cellular and subcellular distribution of proteins and their relative concentrations. In contrast to investigations performed on the DNA or RNA level, proteomics provides the opportunity to study the extent of co- and posttranslational modifications.

Two-dimensional (2D) maps of the major proteins of the different subcellular compartments of host-derived *M. leprae*, with special emphasis on proteins associated with

the cell wall were developed (Marques *et al.* 1998). This revealed the presence of a diverse group of proteins. Immunoblotting and amino acid sequence allowed recognition of the cell wall-associated proteins like the most abundant 65-kDa GroEL-2 protein, a homolog of the *M. tuberculosis* MtrA response regulator protein and EF-Tu. Truncated variants of EF-Tu were also detected by minimal protein identifier (MPI) approach (Mattow *et al.* 2004). This approach takes into consideration that proteins yield characteristic abundant peptides upon proteolytic cleavage and so it facilitated identification of a series of *N*-terminally truncated low-molecular-mass fragments of Mycobacterial Tuf previously not identified by MALDI-PMF. In *M. tuberculosis* were identified fifteen proteins with assigned putative functions, twenty eight conserved hypothetical proteins and one unknown protein by matrix-assisted laser desorption/ionisation and electrospray ionisation mass spectrometry (Mattow *et al.* 2001). Ten protein-coding genes were shown to give rise to more than one spot due to differential co- and posttranslational modifications or cleavage. Comparative proteome analysis of *M. tuberculosis* and attenuated Mycobacterial strains will lead to the identification of *M. tuberculosis* specific low molecular mass proteins, postulated as candidates for vaccine development and diagnosis of tuberculosis.

2.3.5. EF-Tu in Mycobacteria

Elongation factor Tu in Mycobacteria like in other Gram-positive bacteria is encoded by only1 *tuf* gene and was found not only in the cytoplasm, but also in the cell wall of *Mycobacterium leprae*, an obligate intracellular pathogen (Marques *et al.* 1998). Since the cell envelope constitutes the key interface between pathogen and host, the cell wall-associated proteins are presumably crucial determinants of pathogenesis and immunogenicity.

EF-Tu together with electron transfer flavoprotein β-subunit (FixA) and acyl-CoA dehydrogenase (FadE2) was identified in *Mycobacterium avium* within the macrophages (Brunori *et al.* 2004). These proteins may play a role in adaptation and survival of *M. avium* within the macrophages since they were induced intracellularly at an extent higher than in the medium. The strong increase of EF-Tu inside macrophages seems to be related to the macrophage environment. *M. avium* responds to macrophage phagocytosis by upregulating genes involved in biosynthetic and metabolic activities such as EF-Tu, FadE2 and FixA, which may play a role in survival and adaptation to the intracellular life.

EF-Tu was also found to be more abundant in anaerobically growing *Mycobacterium tuberculosis* than in an aerobically growing culture (Starck *et al.* 2004). EF-Tu at dormant state was identified at another spot position in the proteomic map with a higher molecular mass and significantly different pI suggesting some posttranslational modifications of this protein. Since *tuf*1 genes coding for *Streptomyces* and Mycobacterial EF-Tu posses high degree of amino acid sequence identity (up to 86 %) and represent differentiating and non-differentiating *Actinomyces*, we used both proteins in our comparative studies of the role of the protein in the control of their life cycle.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Bacterial strains and plasmids

Mycobacterium smegmatis, Streptomyces granaticolor, Streptomyces coelicolor A3(2), *Streptomyces collinus* and *Streptomyces aureofaciens* 10762 and 84/25, a tetracycline high-producing mutant, were from the collection of microorganisms of the Institute of Microbiology (MBU), Prague. Plasmid bluescript pBS-SK was from the Institute of Microbiology, Bratislava.

3.1.2. Cultivation media

Amounts of all of the ingredients are to prepare 1 l of medium.

MJ

$(NH_4)_2SO_4$	2 g
KH_2PO_4	6.8 g
$MgSO_4.7H_2O$	0.2 g
Glycerol	10 g
Yeast extract	2 g
Casamino acid	10 g
pH 7.2 (using KOH)	

"8"

Yeast extract	4 g
Malt extract	10 g
Glucose	4 g
Agar	20 g
pH 7 – 7.3	

"16"

Sucrose	3 g
Dextrin	15 g
Urea	0.1 g
NaCl	0.5 g
K_2HPO_4	0.5 g
$MgSO_4$	0.5 g
Peptone	5 g
Meat extract	1 g
$FeSO_4$	0.01 g
Agar	30 g
pH 6.5	

Bennet (BM)

Yeast Extract	1 g
Meat Extract	1 g
Maltose	10 g
Trypton	2 g
Agar	15 g
pH 7.2	

LB

Trypton	10 g
Yeast extract	5 g
NaCl	5 g
Thiamine	20 mg
pH 7.1 – 7.2	

7H9 and 7H10

M. smegmatis was grown at 37°C with shaking in Middlebrook 7H9 broth (Difco) containing 0.2% (v/v) glycerol or on solid Middlebrook 7H10 medium (Difco) containing 0.5% (v/v) glycerol.

The media were autoclaved at 120°C for 30 minutes.

3.1.3. Buffers and solutions

Sample buffer for 1D (5x):

1M Tris HCl (pH 6,8)	0.6 ml
Glycerol	2.5 g
10% SDS	2 ml
β– mercaptoethanol	0.5 ml
1% Bromphenol blue	1 ml

Distilled water to 10 ml

Stored at 4°C.

Lysis buffer for 2D:

Urea	12 g
CHAPS	1 g
Pharmalyte 3-10	0.5 ml
DTT	0.25 g
Bromphenol blue	few grains

Add distilled water to 25 ml

Stored at -70°C.

TAE buffer (50X, 1l):

Tris base	242 g
Acetic acid	57.1 ml
$Na_2EDTA.2H_2O$	37.2 g

Binding buffer for GDP binding assay (1l)

1M Tris HCl	10 ml
Magnesium acetate.4 H_2O	2.145 g
β – mercaptoethanol	0.781 g

pH 7.5

Composition of other buffers and solutions is described in the text.

3.2. Methods

3.2.1. Cultivation of *Streptomyces* mycelium in liquid culture

Flasks with liquid medium were inoculated with spores grown on agar plate. After 2 days of aerated cultivation at 28 °C, mycelium was used as inoculum into fresh medium, where it grew for 20 hours. Cells were then centrifuged, washed with standard TMAB buffer (10mM Tris, 10 mM magnesium acetate, 60mM NH_4Cl, 6mM β-mercaptoethanol, pH 7.4) and centrifuged again. Mycelium was stored at –70 °C for later use.

3.2.2. Cultivation of *Streptomyces coelicolor* on glass beads

Two phase cultivation system based on glass beads and supporting differentiation of *Streptomyces* was developed in our laboratory (Nguyen *et al.* 2005). It consists of two components: liquid phase is presented by complex liquid medium MJ and the solid phase is formed by tiny glass beads (Balotina) ranging 325-430 μm in diameter. Balotina, obtained from ORNELA a.s. (Desna in Jizerske mountains, Czech republic), was originally used as the reflective additive in street paints. Before use, glass beads were boiled for 30 min in 0.1M HCl, rinsed many times with water until the washing water became neutral, then with distilled water and dried at laboratory temperature. Just before use, Balotina was sterilized and dried at 120 °C. About 200 g of sterile Balotina was layered into each big Petri dish (14 cm in diameter), which was inoculated with 27 ml of *Streptomyces coelicolor* mycelium culture from exponential phase of growth (20h of cultivation) grown in liquid MJ medium. Plates were then cultivated at 28 °C for proper time.

3.2.3. *Streptomyces* cellular fractionation

The washed harvested mycelium was disintegrated in buffer F containing 50mM Tris.HCl pH 7.4, 10mM magnesium acetate, 10mM β-mercaptoethanol, 100mM KCl and 10μM PMSF and cells were disrupted by SLM.Aminco French pressure cell press. Cell debris was removed by centrifugation in SS34 Sorvall rotor at 9,000 r.p.m and 4°C for 10 minutes and the resulting supernatant was used as "cell free homogenate". Membrane fraction was separated from the cell free homogenate by centrifugation in SS34 Sorvall

rotor at 19,000 r.p.m for 30 minutes. Sedimented pellets were resuspended in a small amount of buffer F and used as a "membrane fraction". The supernatant (S30 fraction) was then laid on 6 ml-cushions of the buffer F containing 10% sucrose and centrifuged at 43,000 r.p.m at 4°C overnight (~16 hours). The supernatant (S150 fraction) contained soluble proteins, and the sediments were used as crude ribosomes.

3.2.4. Purification of *Streptomyces aureofaciens* elongation factor Tu

To prepare chromatography column, the suspension of DEAE-Sepharose CL-6B was reactivated by washing with 1 volume of 1M sodium acetate, pH 3, then with 1.5 volume of 0.5M NaOH over night and then again with 1.5 volume of 1M sodium acetate, pH 3. After each washing it was collected on filter paper and finally it was washed with buffer A (50mM Tris.HCl pH 7.4, 10mM magnesium acetate, 10mM β-mercaptoethanol, 100mM KCl and 10 μM GDP). The suspension was then thoroughly degassed before packing into column, which was equilibrated with 3 volumes of buffer A.

All of the following steps of the procedure were carried out at 4°C to protect protein activity.

About 100 g of frozen cells were resuspended in 150 ml of buffer A supplemented with 10μM PMSF and 0.05 mg/ml bensoase before cell disintegration by passing it through a SLM.Aminco French pressure cell press in 4-5 portions at 1,000-1,500 PSIG. The cell extract was then diluted to 250 ml with buffer A and GDP was added to raise its final concentration to 50μM. After centrifugation at 30,000g for 15 minutes to remove cell debris, the resulting supernatant (S30 fraction) was applied to a column (3x 28cm) of DEAE–Sepharose CL-6B (Pharmacia), which had been equilibrated with buffer A, at the flow rate of 0.6 ml per minute. The system was controlled by the Econo-System of Bio-Rad, which allows buffer gradient mixing and collecting of fractions automatically. The column was then washed thoroughly with 3-5 column volume of buffer A before setting a linear gradient of 0.1-0.4M KCl in total of 1,000 ml buffer A containing 10μM GDP. Elutes were collected in 6ml fractions and the presence of EF-Tu was determined by SDS-PAGE and GDP binding assay. The active fractions were pooled and incubated in 30μM GDP for 30 minutes.

3.2.4.1. Ammonium sulphate precipitation

Proteins tend to aggregate in presence of high salt concentration. Different proteins aggregate in different salt concentrations and so precipitation is often used in protein purification (Scopes *et al.* 1981). During purification of EF-Tu precipitation with ammonium sulphate was used.

Solid ammonium sulphate was added slowly to the final concentration of 56.8 g/100 ml to precipitate total protein. The precipitate was dissolved in 5 ml of buffer A and centrifuged at 18,000g for 10 minutes to remove denatured proteins.

3.2.4.2. Dialysing

Dialysis tubing was boiled in 10mM $NaHCO_3$/1mM EDTA for 10 min, then washed in distilled water, boiled in 1mM EDTA for 10 min and again washed in distilled water.

The solution of proteins was pipetted into dialysis tubing and dialyzed against 2 L of buffer A containing 10μM GDP overnight to remove ammonium sulphate.

The dialyzed solution was then concentrated by immersing the dialysis tubing into dry powder of Sephadex G-200 until the solution had glittered opaque appearance (1-3 ml). After that, the solution was dialyzed again during which all EF-Tu aggregated. Purified EF-Tu was obtained as white sediment by centrifugation of this solution and washing it 3 times with buffer A. The purified aggregated protein can be stored at –70°C for months without loss of activity.

3.2.5. [^{3}H]-GDP binding assay

The activity of EF-Tu was quantified in [^{3}H]GDP exchange assay using filtration on nitrocellulose membranes as described in (Weiser *et al.* 1981). This method uses the fact that EF-Tu binds GDP in ratio 1:1 and so the number of bound GDP molecules corresponds to the number of active EF-Tu molecules. Complex of EF-Tu:[^{3}H] GDPbinds to the nitrocellulose filter, but unbound [^{3}H] GDP flows through. The standard assay mixture contained in 100 μl-volume: 50mM Tris.HCl pH 7.5; 10mM magnesium acetate; 150mM NH_4Cl; 10mM β-mercaptoethanol, 10μM GDP containing 1 μCi of [^{3}H]GDP and 2 – 10 μg of the protein in 10 μl volume.

The filter was washed 3 times with binding buffer and left in a scintillation vial until it dried. 5 ml of scintillation cocktail was added and the sample was counted using an LKB Wallac counting machine for 1 minute.

3.2.6. *In vitro* phosphorylation

Reaction mixtures contained about 50 µg of proteins including a defined kinase source, 50mM Tris.HCl pH 7.4, 50mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 1mM DTT, 10mM $MnCl_2$, 10mM NaF, 1-5 µCi of 10µM[^{32}P] ATP. The reactions were started by adding 10µM ATP containing 1-5 µCi [^{32}P] ATP and kept at room temperature for about 30 minutes, stopped by adding SDS loading buffer (for SDS-PAGE) or by precipitating total protein with cold acetone (for 2-D PAGE).

3.2.6.1. Identification of radiolabelled proteins

Proteins with covalently bound ^{32}P were analysed by electrophoresis and the radioactivity was detected in phosphorimager. Phosphoimaging is in general about 10 times more sensitive than autoradiography. Dried gels were put facing to the active side of a cassette containing Europium based intensifying screen for a proper time (2-7) days. The screen was then scanned with the Fuji phosphorimager Model BAS5000 at the sensitivity of 10,000-30,000, magnitude of 5, resolution of 25-50 µm/pixel.

3.2.7. Determination of protein concentration

Protein concentration was determined using the BCA Protein Assay Kit (Pierce). This method is based on the reduction of Cu^{2+} to Cu^{+} by protein with colorimetric detection of the Cu^{+} by a reagent containing bicinchoninic acid (BCA). The range of sensitivity is 20 µg/ml - 2000 µg/ml of proteins. Solution of bovine serum albumin (BSA) was used as a calibration standard.

A set of diluted BSA standard samples was prepared by diluting the albumin standard in water. Then 50 µl of each standard or sample was pipetted into test tubes and 1 ml of working reagent (working reagent is a mixture of BCA Reagent A and BCA Reagent B (50:1)) was added. The tubes were incubated for 30 min at 37°C and then absorbance was measured in plastic cuvettes at 562 nm against water as a reference. Standard curve was obtained by plotting the blank corrected A_{562} for each of BSA standards against its concentration in µg/µl. Using this standard curve, the protein concentration in the sample was determined.

3.2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used, following the classical procedure described by Laemmli (Laemmli 1970). In this technique proteins are separated according to their molecular weight.

Resolving Gel Solution (10%):	
H_2O	4 ml
30% Acrylamide, 0.8% Bisacrylamide	3.3 ml
1.5M Tris-HCl, pH 8.8	2.5 ml
10% SDS	0.1 ml
10% Ammonium Persulphate (APS)	0.1 ml
TEMED	0.004 ml

Stacking Gel Solution:	
H_2O	2 ml
30% Acrylamide, 0.8% Bisacrylamide	0.5 ml
1M Tris-HCl, pH 6.8	0.38 ml
10% SDS	0.03 ml
10% Ammonium Persulphate (APS)	0.03 ml
TEMED	0.003 ml

TEMED accelerating the polymerisation of acrylamide and bisacrylamide was added at the end, because as soon as it was present in the gel solution, polymerisation begun. The resolving gel solution was poured into the glass plate cassettes and overlaid with isopropanol to prevent oxygen from diffusing into the gel and inhibiting polymerisation. After polymerisation at room temperature was completed (30 min) the overlay was poured off, and the gel was washed with deionised water and dried. Then the stacking gel solution was poured onto the surface of the resolving gel and a comb was inserted in the stacking gel. The gel polymerised in 30 min at room temperature. After that, the comb was removed, the gel cassette was mounted into the electrophoresis apparatus and buffer was added.

Prepared samples were loaded onto the gel (30 µg of proteins per line). SDS-PAGE was performed at 20 – 25 mA per gel.

3.2.8.1. Coomassie Blue Staining

Bacterial proteins were detected by staining with 0.2% (w/v) Coomassie brilliant blue R250 in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 minutes and then quickly destained by boiling in distilled water in microwave (Hervieu 1997) for 2 x 5 minutes. This method is very simple, fast and allows detecting as little as 0.3 µg of protein in a single band/spot.

3.2.8.2. Drying SDS-PAGE gels

Gels were soaked in 2% glycerol solution for 10 minutes and afterwards transferred between two sheets of wet cellophane. The sandwich (cellophane/gel/cellophane) was attached into the drying frame and dried at room temperature overnight.

3.2.9. Two-Dimensional Polyacrylamide Gel Electrophoresis (2D SDS-PAGE)

Two-dimensional gel electrophoresis is a multistep electrophoretic process for resolving of complex mixtures of proteins. The polypeptides are separated according to the independent parameters of isoelectric point (pI) and molecular weight (MW), (O'Farrell 1975). Proteins in a sample are first denatured by dissolving in either urea or SDS buffer. The sample is then applied to the top of a tube gel containing neutral detergent and ampholines forming the pH gradient in the gel or to the prepared IPG strips (see further). Isoelectric focusing (IEF) is carried out as the first-dimension separation to separate proteins according to their own pI. After a brief equilibration in a SDS buffer, the tube gel or strip is transferred to the top of a SDS polyacrylamide slab gel and the proteins are separated according to their MW (SDS-PAGE), (Laemmli 1970). As a combination of these two powerful techniques, two-dimensional gel electrophoresis (2D-PAGE) can separate thousands of polypeptide spots on a single 2D gel as opposed to the 50-band maximum obtained in one dimension alone.

Stock solutions and buffers for the first dimension:

Acrylamide 14.19 g

Bis-acrylamide	0.81 g

Made to 50 ml with distilled water, then filtered and stored at 4°C in the dark.

10% Triton X-100

First dimension sample buffer:

Urea	2.85 g
CHAPS	1 ml of 10% CHAPS stock solution
DTT	80 mg
Sigma Ampholite 5/7	250 µl
Sigma Ampholite 3/10	50 µl

Diluted to 5 ml with distilled water. Stored at -70°C in aliquots of 250 µl.

First dimensional sample overlay buffer:

Urea	2.705 g
Sigma Ampholite	4/6 125 µl
Sigma Ampholite	3/10 25 µl
Bromophenol Blue	250 µl of 0.05% Bromophenol Blue stock solution

Diluted to 5 ml with distilled water. Stored at -70°C in aliquots of 250 µl. Before use dilute 1:1 with distilled water.

Upper chamber buffer:

20mM NaOH

Lower chamber buffer:

10mM H_3PO_4 was thoroughly degassed.

Stock solutions and buffers for the second dimension:

SDS sample equilibration buffer:

Tris.HCl	2.5 ml of 0.5M Tris.HCl, pH 6
SDS	4.6 ml of 10% SDS solution
β-mercaptoethanol	1 ml
Bromophenol Blue	0.5 ml of 0.05% Bromophenol Blue solution

Diluted to 20 ml with distilled water.

SDS running buffer:

Tris	3 g
Glycine	14.4 g
SDS	10 ml of 10% SDS solution

Diluted to 1 L with distilled water.

3.2.9.1. Sample preparation

2 volumes of acetone were added to the protein sample and kept in –20 °C for at least 2 hours. The protein samples in acetone were centrifuged 30 min at 12,000g at 4°C (small laboratory centrifuge MICROSPIN 24S) and acetone was removed. The pellet was resuspended either with one volume of deionised water and supplemented with two volumes of urea sample buffer or with lysis buffer (for IPG strips).

3.2.9.2. First dimension

The first dimension tube gels were cast in 15 cm (1 mm internal diameter) capillary tubes following manufacturer's manual, using a 0.5 cm-diameter casting tube. The protocol for casting tube gels could be varied, depending on different samples and the eventual pH range of interest. The ampholytes were added to the gel solution immediately before polymerising. The gel solution was degassed prior to the addition of the catalysts (APS and TEMED) and left to polymerise for 1 hour. The tube gels were assembled in the first dimension apparatus as described by the manufacturer. Degassed 20mM NaOH solution was used as catholyte and degassed 10mM H_3PO_4 solution was used as the anolyte. Proteins were loaded at the cathode on the first dimension gel and electrophoresed at 200 V for 15 minutes and then 400 V for 16 hours. The electrophoresis times should be determined empirically for specific samples. In some cases, voltage was increased to 750 V for the last hour of the run.

1st dimensional gels preparation:

Urea	2 g
Distilled water	1.575 ml
Acrylamide solution	500 µl
CHAPS	0.1 g

Degassed about 5 minutes.

Sigma Ampholite 5-7	100 µl
Pharmacia Ampholite 4-6	100 µl
Sigma Pharmalite 3-10	50 µl
APS	18 µl
TEMED	5 µl

After mixing these components gave about 4 ml of total volume, enough to cast 8 gels of 1 mm-diameter and 15 cm height. The mixture should be made fresh. Urea was dissolved by warming and swirling in hands. Ampholites and catalysts were added at the end and the gel solution was gently but quickly swirled before casting the gels.

Running condition:

Prefocussing was done for 30 minutes at constant 500 V voltage with 50 µl of 2X diluted sample buffer, current and wattage were set to maximum of 10 mA and 10 W, respectively. After prefocussing, the tops of gels were washed with 50 µl of distilled water and then with 50 µl of 2X diluted sample buffer before loading samples. The system was cooled at 15°C.

Focussing was run for a total 14,000 V/hr in total 17 hours running, with following steps:

500 V for 1 hour

800 V for 15 hours

1,500 V for the last 1 hour

Maximum current and wattage were set the same as those used for prefocussing and should gradually decrease during run.

After the first dimension, the tube gels were extruded onto the layer of a parafilm and equilibrated in SDS equilibration buffer for 10 minutes before transferring to the top of either 10% or 12% SDS acrylamide slab gel. The second dimension slab gels were prepared following the standard procedure of Laemmli (Laemmli 1970), (see further).

IPG strips

For the isoelectric focusing non-linear immobilised pH gradient (IPG) strips (Amersham), 18 cm long with a pH range from 4 to 7, were used. Prior to the first dimension run, the IPG strips together with the samples were rehydrated using the following method (in-gel sample rehydration): IPG strips were placed in a reswelling tray. Protein samples were diluted to 360 µl with lysis buffer and pipetted into the middle of a reswelling tray chamber. After removing the protective cover from the IPG strip, the strips were put gel side down into the chamber, the chamber was filled with 2 ml of mineral oil and left overnight.

The first dimension was run on a horizontal electrophoresis unit at 20°C for 26.5 hours using the following running conditions:

Step 1 150 V 1 mA 5 W 1 Vh

Step 2 150 V 1 mA 5 W 300 Vh 2 h

Step 3 300 V 1 mA 5 W 1 Vh

Step 4 300 V 1 mA 5 W 600 Vh 2 h

Step 5 3500 V 1 mA 5 W 10 kVh 5 h

Step 6 3500 V 1 mA 5 W 60 kVh 17.5 h

Equilibration of IPG strips

Stock solutions:

Equilibration solution 1: 50mM Tris HCl, 6M Urea, 30% Glycerol, 10% SDS, just before use 0.2 g DTT/10 ml equilibration solution was added.

Equilibration solution 2: 50mM Tris HCl, 6M Urea, 30% Glycerol, 10% SDS, just before use 0.25 g Iodoacetamide/10 ml equilibration solution was added.

0.5M Tris HCl, pH 6.8 (stored at 4 °C)

When the isoelectric focusing was finished, the IPG strips were placed into Petri dish, 15 ml of equilibration solution "1" per strip was added and Petri dish was shaken for 10 min. Then equilibration solution "1" was replaced with equilibration solution "2" and dishes were shaken for another 10 min. After that, the samples were loaded onto the SDS-PAGE gels.

3.2.9.3. Second dimension

Stock solutions:

30% Acrylamide, 0.8% Bisacrylamide (stored at 4 °C)

1.5 M Tris (stored at 4 °C)

10% SDS: (stored at room temperature)

10% Ammonium Persulphate: (stored at 4 °C)

2D-gel running buffer: 192mM Glycine, 25mM Tris, 0.1% SDS (stored at 4 °C)

Overlay agarose: 2D-gel running buffer, 0.5% Agarose

Equilibration buffer: 125mM Tris HCl, 20% Glycerol, 3% SDS, Bromphenol blue (stored at room temperature); just before use 0.23 g DTT was dissolved in 30 ml of this buffer.

1% agarose in equilibration buffer: (stored at 4 °C)

The following gel solution composition was used to prepare 6 gels 1 mm thick (21 cm x 22 cm in size).

Slab gel solution (12.5%)

30% Acrylamide, 0.8% Bisacrylamide	83 ml
1.5 M Tris	50 ml
dd H_2O	64.4 ml
10% SDS	2 ml
10% APS	500 µl
TEMED	100 µl

The gel solution was poured into the glass plate cassettes, overlaid with 200 µl of isopropanol and let to polymerise at room temperature. When polymerisation was completed (after 2 hours) isopropanol was washed off with distilled water and let to dry. Equilibrated IPG strips were placed on the top of SDS-PAGE gels and overlaid with melted agarose. Gel sandwiches were then transferred into precooled vertical electrophoresis unit containing 10 l of 1X 2D-gel running buffer. The upper buffer chamber of the electrophoresis unit was filled with 3 l of 2X 2D-gel running buffer and the gels were run at cca 90 mA per gel for about 4h 30 min.

The purified EF-Tu standard was dissolved in equilibration buffer with 1% agarose (10 µl EF-Tu and 150 µl agarose) and casted into glass capillary. The agarose gel piece (0.5 cm) was applied on the top of SDS-PAGE gel after placing there the IPG strip.

3.2.10. Silver Staining

For silver staining of proteins the procedure of (Rabilloud 1992) was followed. Using this technique as little as 2 ng of protein in a single spot can be detected.

Reagents:

Fix solution 1:

Ethanol	400 ml
Acetic acid	100 ml
Deionised water to 1 l	

Fix solution 2:

50% glutaraldehyde	10 ml
Ethanol	300 ml
Potassium tetrathionate	2.5 g
Sodium acetate	68 g
Deionised water to 1 l	

Silver nitrate solution:

Silver nitrate	2 g
Formaldehyde	0.25 ml
Deionised water to 1 l	

Developer solution:

Potassium carbonate	30 g
Sodium thiosulphate	7.5 mg
Formaldehyde	0.15 mg
Deionised water to 1 l	

Stop solution:

2% acetic acid

Glycerol solution:

2% glycerol

Gels were fixed in solution "1" for 1 hour and then placed into fix solution "2" for 1 hour up to overnight. After that, the gels were washed 4 times for 15 min with deionised water and placed into silver solution for 30 min. Then they were washed for 1 minute in deionised water, placed in a developer solution for 5 to 30 min (until protein spots were visible) and transferred to a stop solution for 10 minutes. Thereafter, the gels were put in glycerol solution for 10 minutes and dried as described in chapter 3.2.8.2. All steps were performed at room temperature while gently agitating the trays with gels on a rotating platform.

3.2.11. Increasing the specific activity of EF-Tu polyclonal antibody

Drops of EF-Tu (20 µg in 20 µl volume) were applied on nitrocellulose membrane (0.5 x 7 cm). After drying, it was blocked with 5% non-fat dry milk in TBST (20mM Tris-HCl pH 7.6, 137mM NaCl, 0.1% Tween 20) for 1 h at laboratory temperature. After 3 times washing with TBST (for 10 min) membranes were incubated overnight at 4 °C with serum raised in rabbits immunized with purified EF-Tu. Serum was diluted with TBST in ratio 1:5 and incubation was performed in plastic bag to allow binding of anti EF-Tu antibody to EF-Tu absorbed in nitrocellulose membrane. Afterwards membranes were washed 3 times with TBST at laboratory temperature and bound antibody was finally released by 0.2M glycine (pH 2.8), 1mM EDTA for 10 min and neutralized with 1M Tris HCl (pH 9).

3.2.12. Western Blot and Immunodetection

Nitrocellulose membrane was first incubated for 5 min in distilled water and then at least for 5 min in CAPS buffer (0.22% CAPS, 10% methanol, pH 11). Protein spots from 2-D gels or bands from SDS-PAGE gels were transferred onto these membranes in a semi-dry blot cell (BioRad) following the manual instructions. Transfer took from 2 hours up to overnight.

For EF-Tu detection, the membranes were blocked with 5% non-fat dry milk in TBST (20mM Tris-HCl pH 7.6, 137mM NaCl, 0.1 % Tween 20) for 1 h at laboratory temperature. After 3 times washing with TBST (1st time for 15 min and then 2 times for 5 min) blots were incubated with primary EF-Tu antibody at laboratory temperature for 1 hour up to overnight at 4 °C. After washing in TBST, 2nd anti-rabbit antibody with conjugated

horseradish peroxidase (Amersham) in TBST was added to blots, incubated for 1 h at laboratory temperature and afterwards blots were again washed with TBST.

For detection of P-Ser or P-Thr proteins, the membranes were blocked with 10% BSA in TBST containing 0.1 % Tween 20 for 2 hours at laboratory temperature or overnight at 4 °C. Blots were incubated with primary anti P-amino acid monoclonal antibody at laboratory temperature for 2 hours or overnight at 4 °C. Anti-mouse 2nd antibody with conjugated horseradish peroxidase (Amersham) in TBST was added to blots and incubated for 1 h at laboratory temperature. Blots were also washed with TBST buffer between the steps.

West pico Western Blot reagents (Pierce) were used for the detection following the manufacturer's instruction.

3.2.13. *In vitro* DNA manipulations

All restriction, endonuclease digestions, ligations, DNA modifications and PCR amplifications were performed according to standard protocols (Sambrook and Russell 2001).

Oligonucleotides tufsa2 (5′CCCCGGATCCTCGTGGGCCTTCGGACGAGCC) and tufsa4 (5′CCCCGGATCCCGGGCGACGACCTGCCGGTCG); tufsa3 (5′CCCCGGATCCTTGTCGCCCTCGAGGGCC) and tufsa5 (5′CCCCGGATCCAGTGGCCAAGGCGAAGTTCGAGCGG), *Bam*HI sites underlined, were used to amplify 760bp and 700bp DNA fragments respectively of *tuf*1 gene coding for active EF-Tu. These fragments were cloned into pBS-SK plasmid, which was then transformed into *E. coli*, isolated and sequenced.

3.2.13.1. PCR (Polymerase chain reaction) cycle

Reaction mix:

10X PCR buffer (without Mg^{2+})	2 µl
25mM $MgCl_2$	2 µl
DNA (4 ng/µl)	1 µl
Oligonucleotide 1 (2.5 pmol/µl)	0.6 µl
Oligonucleotide 2 (2.5 pmol/µl)	0.6 µl
2.5 mM dNTP	0.8 µl

Taq polymerase (5 units/µl)	0.3 µl
deionised and autoclaved water	12.7 µl

PCR cycle (DG):

1) 97 °C 5 min
2) 97 °C 30 s
3) 35 °C 30 s
4) 72 °C 2 min
5) GO TO 2) 4x
6) 97 °C 30 s
7) 57 °C 30 s
8) 72 °C 2 min
9) GO TO 6) 29x
10) 72 °C 5 min
11) 4 °C until removing the samples

3.2.13.2. Bacterial transformation

E. coli XL1 Blue was transformed by the method of temperature shock. Competent cells were mixed with 1/10 of plasmid DNA and incubated on ice for 40 min. After that cells were transferred to 42 °C for 40 s, then cooled and mixed with LB medium. After incubation at 37 °C for 1 hour they were sowed on agar medium LB with ampicilin, IPTG and X-gal for blue-white selection.

Competent *E. coli* cells were prepared in TB solution (10mM PIPES, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl) by adding DMSO to final concentration 7%, cooled on ice and frozen in liquid nitrogen.

3.2.13.3. Preparation of genomic DNA from *S. aureofaciens*

Mycelium was resuspended in 5 ml of SET buffer (75mM NaCl, 25mM EDTA pH 8, 20mM Tris-HCl pH 7.5). Afterwards lysozyme was added to final concentration 1mg/ml and solution was incubated at 37 °C for 30 – 60 min. Thereafter proteinase K solution was added to final concentration 0.5 mg/ml and 600 µl of 10% SDS, mixed and incubated for 2 hours at 55 °C. Then 2 ml of 5M NaCl were added, mixed and let cool to 37 °C. After that the solution was extracted first with alkaline phenol-chlorophorm (1:1) until disappearance

of an interphase, then with chlorophorm-izoamylalkohol (24:1). All extractions were with ratio 1:1. Afterwards the solution was dried from chlorophorm and RNase was added to final concentration 100 µg/ml and incubated for 1 hour at 37 °C. Then the solution was precipitated with 1/10 volume of 3M sodium acetate and 0.54 volume of isopropanol, DNA was spooled onto a sealed Pasteur pipette, rinsed in 70% ethanol, air dried and dissolved in 3 to 4 ml of TE buffer (2mM EDTA, 10mM Tris-HCl, pH 8) at 50 °C.

Then SDS and proteinase K solution were added, incubated at 50 °C for 1 hour, extracted again with phenol-chorophorm and chlorophorm, precipitated and at the end resuspended in a proper amount of TE buffer.

3.2.13.4. Mini screen

1.5 ml of cells from liquid medium was resuspended in STET buffer (8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris pH 8). 20 µl of lysozyme solution (10 mg/ml) was added and the mixture was incubated for 5 min at laboratory temperature, then boiled for 2 min. After centrifugation, supernatant was precipitated with 500 µl of 2.5M ammonium acetate and 1 volume of 75% isopropanol, incubated for 20 min at laboratory temperature and centrifuged. Pellet was washed with 70% ethanol, dried and dissolved in TE buffer.

A part of this solution was digested with *Bam*HI to see whether it contained our fragment of *tuf*1 gene.

3.2.13.5. Preparation of plasmid DNA

Plasmid DNA was prepared by the method described in QIAGEN Plasmid Purification Handbook.

3.2.13.6. DNA electrophoresis

Electrophoresis was performed in 1% agarose gel in TAE buffer with constant voltage 50V.

4. RESULTS

4.1. Functional and structural aspects of elongation factor Tu

4.1.1. Two strains of *Streptomyces aureofaciens*

We isolated EF-Tu from two closely related strains of *Streptomyces aureofaciens.*

Streptomyces aureofaciens 84/25 tetracycline-producing strain was used as a model strain in the Institute of Microbiology. It is not the direct descendent of the wild type *Streptomyces aureofaciens* and *its* origin is not known.

On the other hand, *Streptomyces aureofaciens* ATCC 10762 could be considered as a wild type *Streptomyces aureofaciens.* This strain was isolated from the soil and described by Duggar (USA) in 1949 and so it is well defined. The rationale for this strategy was, that although there exist minor differences in the primary sequence of *Streptomyces* EF-Tus they often differ in some of their properties, such as apparent differences in SDS electrophoresis determined molecular mass or abilities for spontaneous aggregation of the protein (Nguyen 2001).

4.1.2. Purification of EF-Tu

EF-Tu was purified from two above mentioned strains by the method using spontaneous aggregation of the proteins (Weiser *et al.* 1982). The EF-Tu during the purification procedure was detected by SDS-PAGE (Fig. 6A), confirmed by immunodetection with anti EF-Tu antibody (Fig. 6B) and its activity was measured by GDP binding assay (Fig. 7).

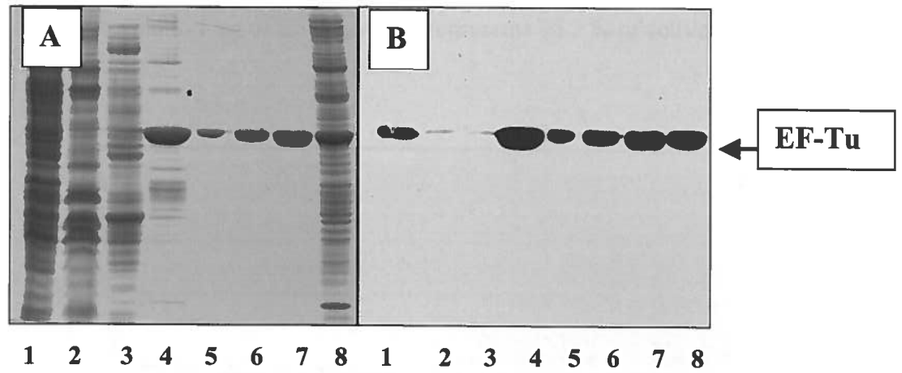


Figure 6. Analysis of the purification of aggregated EF-Tu.GDP from *S. aureofaciens* 84/25 by SDS-Page. (A) Gel stained with Coomassie blue, (B) immunodetection with polyclonal antibody against EF-Tu. Analysed samples: (1) cell free extract prior applying on the DEAE Sepharose column, (2) fraction which did not bind to the column, (3) fraction released by high salt after chromatography, (4) pooled fractions containing active EF-Tu, (5 – 7) 1, 2 and 4 μg of purified EF-Tu respectively and (8) supernatant after sedimentation of aggregated protein.

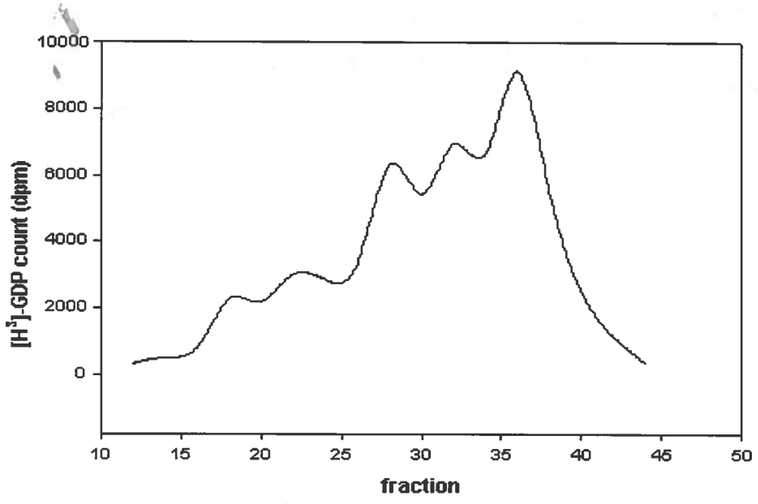


Figure 7. Presence of EF-Tu in fractions eluted from DEAE Sepharose CL-4B column confirmed by GDP-binding activity. Strain *S. aureofaciens* 84/25.

The activity of EF-Tu was determined by GDP binding, since it is proportional (1:1 ratio) to the amount of active factor. The amount of bound GDP is easily detected in a scintillation counter after exchange reaction with [^{3}H] GDP. Specific activity of purified

protein was deducted from the calibration curve (results not shown) and it was 0.0097 µg of GDP bound to 1 ug of EF-Tu, which represents 95.2 % of active EF-Tu.

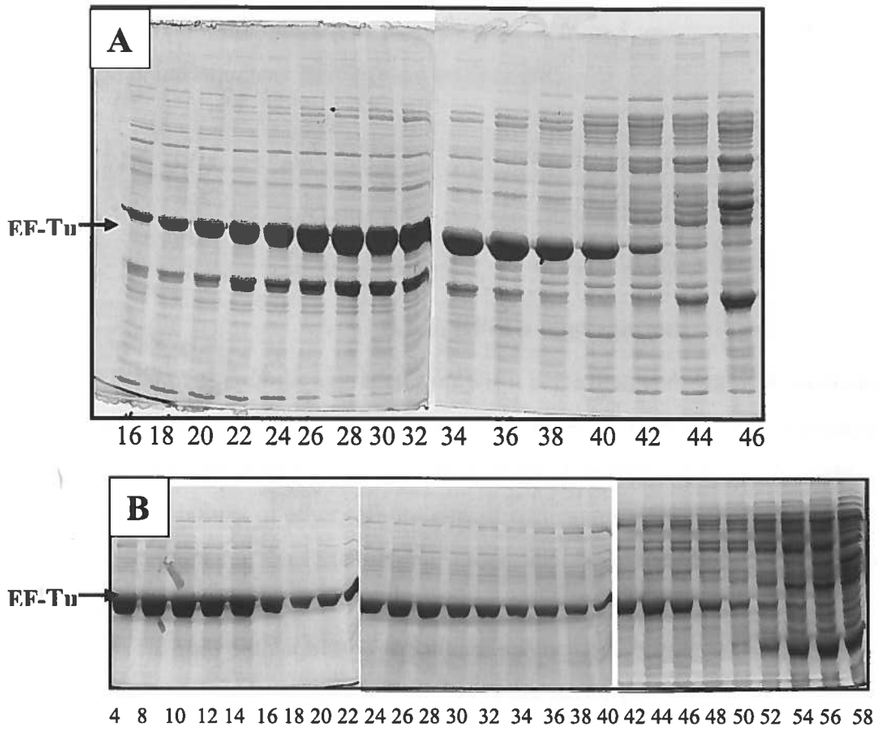


Figure 8. SDS-PAGE was used to detect the presence of EF-Tu in fractions eluted from DEAE Sepharose CL-4B column. Purification of EF-Tu from *S. aureofaciens* 84/25 (A) and *S. aureofaciens* 10762 (B). Fractions are numbered respectively.

We found that EF-Tu from the wild type *Streptomyces aureofaciens* 10762 is also capable of spontaneous aggregation and so we could purify it using the same method (Weiser *et al.* 1982). Moreover, we found that purification of the factor from this strain is even more effective, since we gained relatively greater yield of EF-Tu after ion-exchange chromatography. There were almost no other proteins in fractions containing EF-Tu (Fig. 8). Also the aggregation of the protein during the last step of the purification procedure was much more effective.

Figure 9. SDS-Page of 2 µg of purified EF-Tus from *S. aureofaciens* 84/25 (band on the left) and *S. aureofaciens* 10762 (band on the right).

4.1.3. EF-Tu aggregation

EF-Tu of *S. collinus* aggregates only if it is purified and that of *S. coelicolor* does not aggregate at all under physiological conditions (Weiser, personal communication). Since it was observed that only EF-Tu from *Streptomyces aureofaciens* 84/25 readily aggregates even in the mixtures of other cell proteins, it was examined whether it differs in this ability together with some basic structural properties from the close relative *Streptomyces aureofaciens* 10762.

EF-Tu from both strains is capable of spontaneous aggregation. It forms filamentous structures, which were observed in the light microscope (Fig. 10).

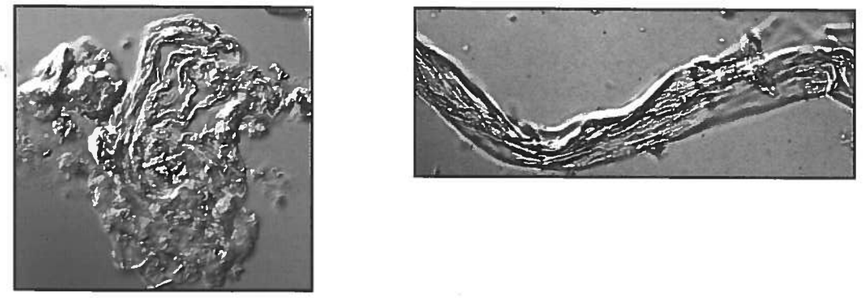


Figure 10. Aggregates of EF-Tu from *S. aureofaciens* 84/25 (on the left) and EF-Tu form *S. aureofaciens* 10762 (on the right) under the light microscope OLYMPUS BX-60, objective with olive inversion 100X/1.4. Pictures were captured with CCD camera Fluoview™. Magnification 100X.

4.1.4. 2D analysis of EF-Tu

Purified EF-Tus from both *S. aureofaciens* strains were analysed by 2D gel electrophoresis. There were detected usually three isoforms of the protein in purified EF-Tu preparations in both strains. In some cases also a multimer of EF-Tu was detected (see Fig. 11). pH range of the gels was 4 – 7 (from left to right) and 12% acrylamide was used in slab gels. 2.5 μg per gel was sufficient concentration of the protein amount. These isoforms represent posttranslational modification of EF-Tu and their distribution is in the Fig. 12. In order to identify the nature and location of posttranslational modification we asked mass spectrometry lab in the Institute to analyze individual isoforms by FT-MS (fourier transform mass spectrometry). The experiments are currently under way.

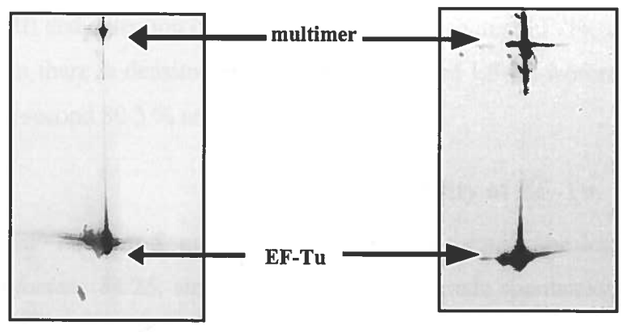


Figure 11. EF-Tu from *S. aureofaciens* 84/25, silver stained gel (on the left) and detection of EF-Tu with antibody against EF-Tu (on the right), pI 4 – 7. Multimer of EF-Tu is at the top of the gel.

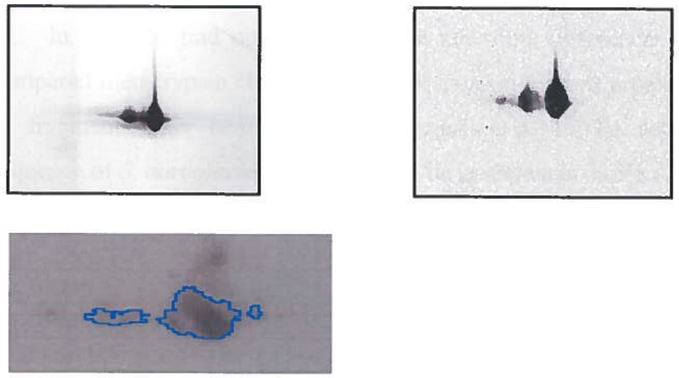


Figure 12. At the top is EF-Tu from *S. aureofaciens* ATCC 10762, silver stained gel (on the left) and detection of EF-Tu with antibody against EF-Tu (on the right), pI 4 – 7. At the bottom there is densitometry pattern of purified EF-Tu isoforms. The first spot represents 17 %, second 80.5 % and third 2.5 %.

4.1.5. Stability of EF-Tu

EF-Tu from *S. aureofaciens* 10762 was found to be less stable than EF-Tu from *S. aureofaciens* 84/25, since it was found to degrade spontaneously into one large fragment after 1-hour cultivation at 37 °C (Fig. 13).

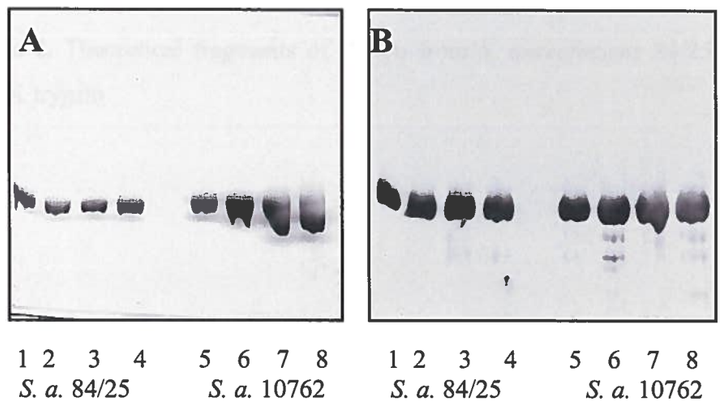


Figure 13. Incubation of EF-Tu at 37 °C monitored by electrophoresis after 0 min (1,5), 30min (2,6), 1 h (3,7), 2 h (4,8), SDS – Page (A), Western blot (B).

4.1.6. Digestion of EF-Tu by TPCK trypsin

In order to find out, whether there are some differences between two factors, we compared their trypsin cleavage products. Trypsin cleaves proteins behind Lys or Arg and so fragments after complete trypsin digestion of EF-Tu determined from nucleotide sequence of *S. aureofaciens* 84/25 should be as shown in Table. 2.

mass	position	peptide sequence
4190.0262	338-377	TTDVTGVVTLPEGTEMVMPGDNTDMTVALIQPVAMEEGLK
2701.3357	93-119	NMITGAAQMDGAILVVAATD GPMPQTK
2656.4344	293-317	GQVIIKPGSVTPHTEFEAAA YILSK
2434.2387	158-179	ELLSEYDFPGDDLPVVQVSA LK
2181.0896	208-226	DTDKPFLMPVEDVFTITGR
2117.0318	140-157	ADMVDDEEILELVELEVR
2033.9769	323-337	HTPFFNNYRPQFYFR
1823.9874	9-25	TKPHVNIGTIGHIDHGK
1807.9622	191-207	LLGLMDAVDEAIPTPPR
1787.9286	62-77	GITISIAHVEYQTEAR
1725.7802	78-92	HYAHVDCPGHADYIK
1696.9228	268-283	LLDEGQAGENVGLLLR
1667.7911	40-54	YPDLNAASAFDQIDK
1399.8307	127-139	QVGVPYIVVALNK
1356.6828	255-266	TTTTVTGIEMFR
1200.6834	241-251	VNETVDIIGIK

Table 2. Theoretical fragments of EF-Tu from *S. aureofaciens* 84/25 after digestion by TPCK trypsin

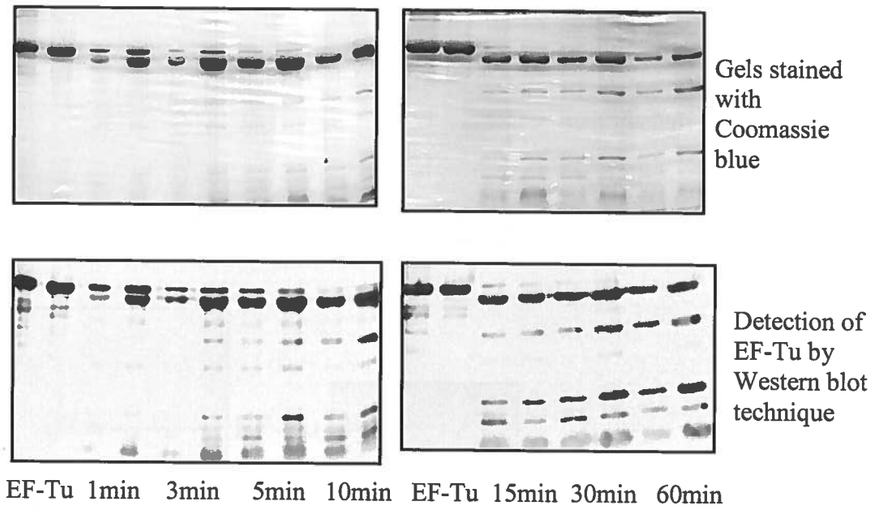


Figure 14. Limited trypsin digestion of EF-Tu from *S. aureofaciens* 84/25 (band always on the left) and from *S. aureofaciens* 10762 (band always on the right). First two bands are intact EF-Tus from either of the strains.

EF-Tu was incubated with TPCK trypsin at 30 °C for different times (see Fig. 14). Samples were loaded on SDS-PAGE (Fig. 14) or 2D gel system (Fig. 16). EF-Tu from *S. aureofaciens* 10762 seemed again less stable than that of *S. aureofaciens* 84/25, especially in short periods of time, since more fragments and less intact EF-Tu were detected. This led us to an attempt to compare primary sequences of these two proteins (see further).

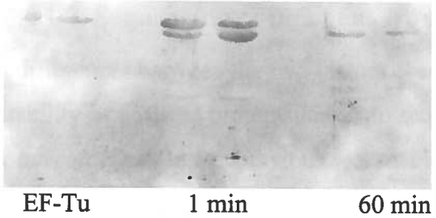


Figure 15. Detection of digested EF-Tu from both strains of *S. aureofaciens* by monoclonal antibody against phosphothreonine.

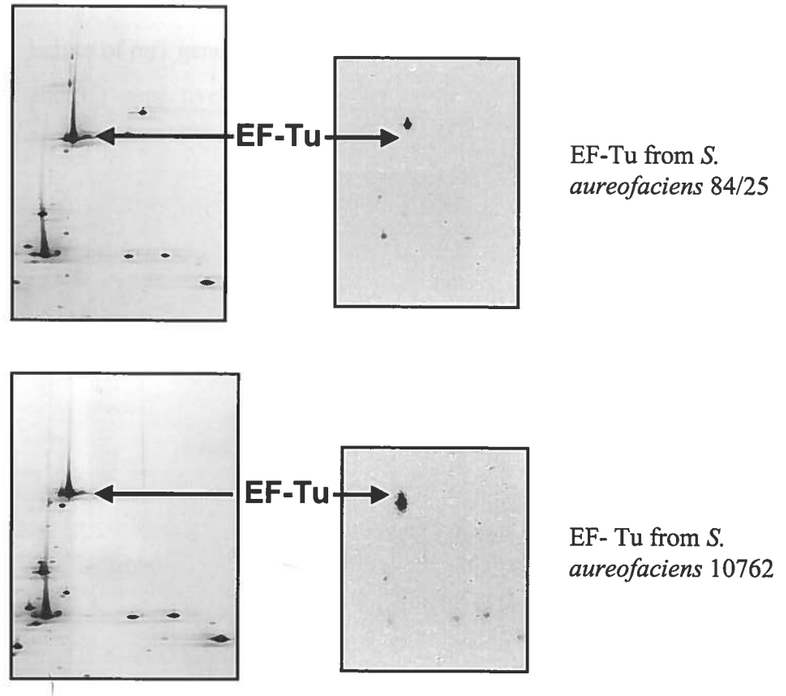


Figure 16. EF-Tu on 2D gel digested 1 hour with TPCK trypsin, 20 µg of EF-Tu per gel.

EF-Tu trypsin fragments detected by 1D and 2D electrophoresis were the same from both strains and were detected also by antibody raised against this protein. First two fragments were also detected by monoclonal antibody against phosphothreonine (Fig. 15), which means that these contain phosphorylated Thr residue.

4.1.7. Sequencing of *tuf*1 gene from *S. aureofaciens* ATCC 10762

Slight differences between the two factors observed during purification procedure and in protein stability of purified proteins led us to sequence comparison of EF-Tus from two strains of *S. aureofaciens*. In our previous work we sequenced *tuf*1 gene from *Streptomyces aureofaciens* 84/25 coding for active EF-Tu (Kormanec *et al.* 2001). Since we expected high homology of *tuf*1 genes from both *S. aureofaciens* strains, oligonucleotides for PCR reactions were designed based on known sequence from *S. aureofaciens* 84/25. In the Fig. 17 are the results of PCR amplification using oligonucleotides as follows: A: tufsa5 + tufsa2, B: tufsa5 + tufsa3, C: tufsa2 + tufsa4, see chapter 3.2.13. for details. It was not possible to amplify the whole gene (Fig. 17 (A)) so the gene was divided into 2 parts. Two

halves of *tuf*1 gene were amplified and we gained fragments of 700 and 760 bp (Fig. 17 (B) and (C) respectively).

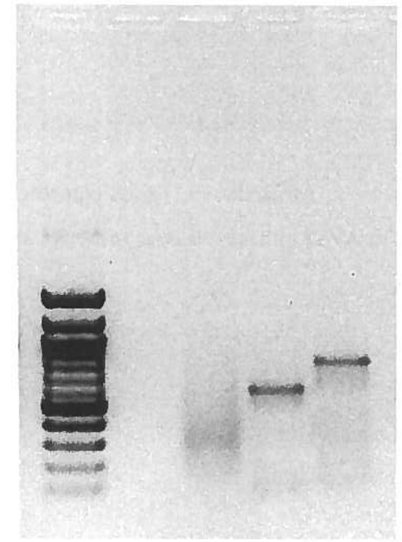
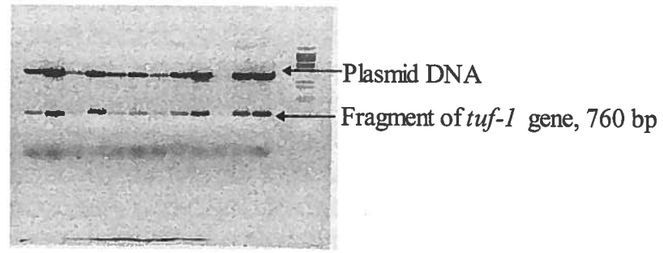


Figure 17. PCR amplification using oligonucleotides A: tufsa5 + tufsa2, B: tufsa5 + tufsa3, C: tufsa2 + tufsa4.

The fragment of 700 bp was isolated and sequenced directly, the fragment of 760 bp was cloned into the plasmid and thereafter sequenced. The place of insertion in the plasmid was gene coding for β-galactosidase. Only positive clones with disrupted gene are therefore white. We checked presence of our fragment in all white transformed cells by BamH1 digestion and concluded that all clones contained our fragment (Fig. 18). After that we isolated our plasmid with the second part of *tuf*1 gene, presence of which was afterwards once more confirmed by BamH1 digestion (Fig. 19 – (2), where split of fragment of *tuf*1 is shown).



transformed cells standard

Figure 18. Mini screen (plasmid DNA of transformed cells digested by BamH1).

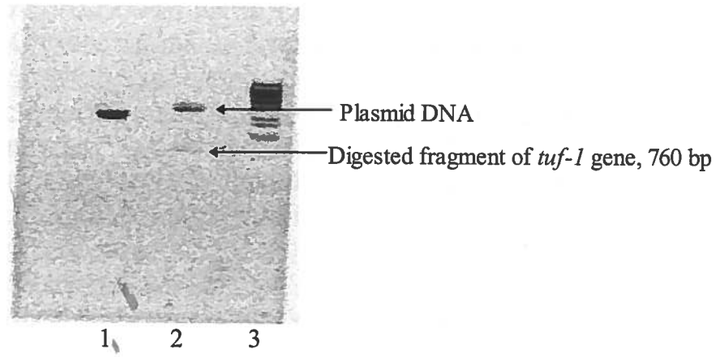


Figure 19. Purified plasmid with cloned part of *tuf*1 gene. (1) whole plasmid DNA, (2) plasmid DNA digested by BamH1, (3) standard

Nucleotide sequence of *tuf*1 gene from *S. aureofaciens* 10762 is in Table 3.

ATTGCCAAGGCGAAGTTCGAGCGGACGAAGCCCCACGTCAACATCGGCACCA
TCGGTCACATCGACCACGGCAAGACCACGCTGACCGCGGCCATCACCAAGGT
GCTGCACGACGCGTACCCGGAGATCAACCCCTTCACGCCGTTCGACCAGATC
GACAAGGCCCCGGAGGAGCGTCAGCGCGGTATCACCATCTCGATCGCGCACG
TCGAGTACCAGACCGAGGCGCGTCACTACGCCCACGTCGACTGCCCGGGTCA
CGCTGACTACATCAAGAACATGATCACCGGTGCCGCCCAGATGGACGGCGCC
ATCCTGGTGGTCGCCGCCACCGACGGCCCGATGCCGCAGACCAAGGAGCACG
TCCTCCTGGCCCGCCAGGTCGGCGTTCCGTACATCGTCGTCGCCCTGAACAA
GGCCGACATGGTGGACGACGAGGAGATCCTGGAGCTCGTCGAGCTCGAGGTC
CGCGAGCTCCTCTCGGAGTACGAGTTCCCGGGCGACGACCTGCCGGTCGTCC
GCGTCTCCGCCCTGAAGGCCCTCGAGGGCGACAAGGAGTGGGGCGAGAAGCT
CCTCGGCCTCATGCACGCGGTCGACGAGAACATCCCCACCCCGGCCCGCGCC
GTGGACCAGCCGTTCCTGATGCCGATCGAGGACGTCTTCACGATCACCGGTC
GTGGCACCGTCGTCACCGGTCGTATCGAGCGCGGCATCCTCAAGGTCAACGA
GACCGTCGACATCATCGGCATCAAGACCGAGAAGACCACCACCACGGTCACC
GGCATCGAGATGTTCCGCAAGCTGCTCGACGAGGGCCAGGCCGGTGAGAACG
TCGGTCTGCTGCTCCGTGGCATCAAGCGCGAGGACGTCGAGCGCGGCCAGGT
CATCATCAAGCCGGGTTCGGTTACCCCGCACACCGACTTCGAGGCCCAGGCC
TACATCCTGTCGAAGGACGAGGGTGGCCGCCACACCCCGTTCTTCAACAACT
ACCGCCCGCAGTTCTACTTCCGTACCACGGACGTGACCGGCGTCGTGACCCT
CCCCAAAGGCACCGAGATGGTCATGCCGGGCGACAACACCGCCATGACCGTC
GCGCTGATCCAGCCCGTCGCCATGGAGGAGGGCCTGAAGTTCGCCATCCGTG
AGGGTGGCCGTACCGTCGGCGCCGGCCAGGTCACCAAGATCGTCAAG

Table 3. Nucleotide sequence of *tuf*1 gene from *S. aureofaciens* 10762. Nucleotides, which differ from *tuf*1 gene of *S. aureofaciens* 84/25 are in blue.

Even though there is a number of differences in nucleotide sequence of *tuf*1 gene from *S. aureofaciens* 84/25 and 10762, deduced amino acid sequences are identical (Table 5).

4.1.8. Comparative modelling of EF-Tu

Obtaining the sequence of *tuf*1 gene coding for active EF-Tu in *Streptomyces aureofaciens* 10762 permitted us to create and compare 3D model structures of EF-Tu from *S. aureofaciens* with those of *Streptomyces coelicolor* and *Mycobacterium smegmatis* factors (Figs. 20, 21 and 22). Crystal structure of EF-Tu from *E. coli* (Song *et al.* 1999) has been used as a template to build homology models of EF-Tu from *S. aureofaciens, S.*

coelicolor and *M. smegmatis* using the program Deep-View/Swiss-PbdViewer (Peitsch 1995; Guex and Peitsch 1997; Schwede *et al.* 2003).

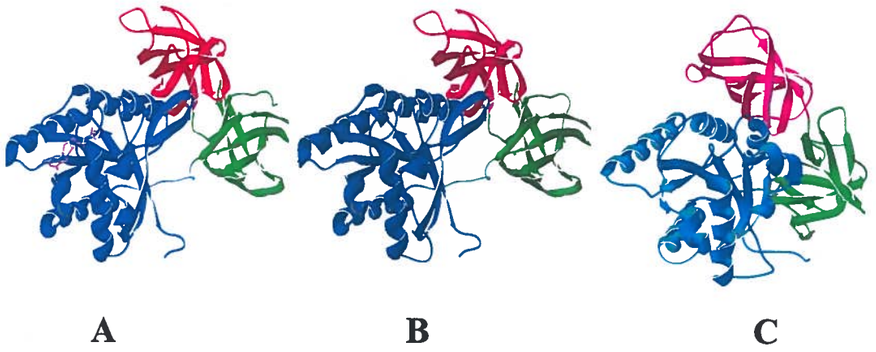


Figure 20. 3D models of EF-Tu from *Streptomyces aureofaciens* (A) with GDP ligand (pink), *Streptomyces coelicolor* (B) and *Mycobacterium smegmatis* (C). First domain is blue, second green and third red.

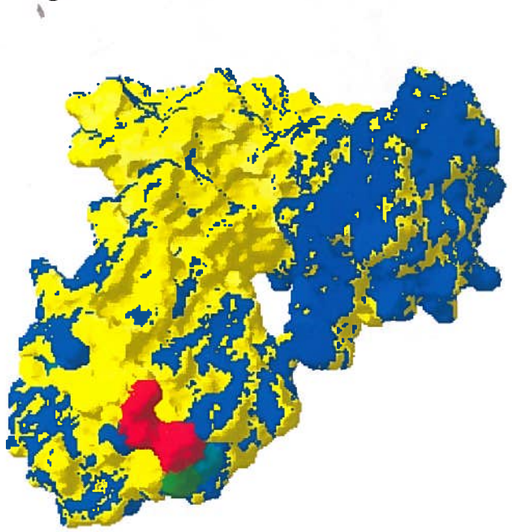


Figure 21. Comparison of *S. coelicolor* EF-Tu molecule surface (blue) with that of *S. aureofaciens* (yellow). Most different areas are red and green.

We found that certain small part of *S. aureofaciens* and *S. coelicolor* EF-Tu surface structures differs, which might be a ground for differences in aggregation. Different amino acids are listed in Table 4.

S. aureofaciens	Glu 42	Ile 43	Asn 44	Pro 45	Phe 46
S. coelicolor	Asp 42	Ile 43	Asn 44	Glu 45	Ala 46

Table 4. Amino acids in a distinguished red region of EF-Tu from *Streptomyces*.

Since amino acid sequence of EF-Tu from Mycobacteria is much more different from EF-Tu of Streptomycetes (Table 1), the differences between the 3D structure of *Streptomyces* EF-Tu and that of *M. smegmatis* are much more pronounced (Fig. 22).

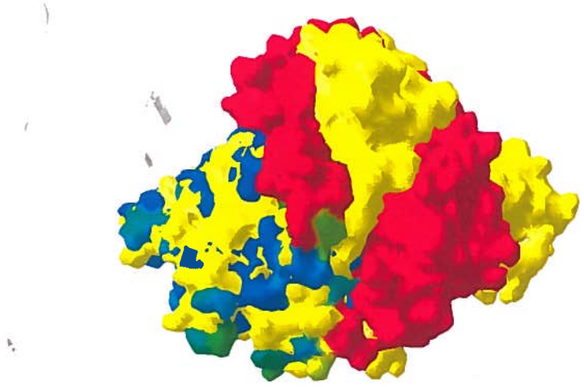


Figure 22. Comparison of *S. aureofaciens* EF-Tu molecule surface (yellow) with that of *M. Smegmatis* (blue). Most different areas are red and green.

Since amino acid sequence of *M. smegmatis* EF-Tu differs more from that of EF-Tu from *Streptomyces* it is difficult to fit structures on top of each other. The red part in Fig. 22 represents areas of the protein, which did not fit well in backbones of peptides and in the surfaces as well, even though some sequences in these regions are identical.

Comparing molecules between two *Streptomyces* was easier, since amino acid sequences are almost identical. Molecules do not fit to each other especially in regions, where there are different amino acids of different properties. Since *M. smegmatis* EF-Tu

has many different amino acids in various parts of the protein, fitting of molecules is easier in some parts (similar amino acid sequences) than in the others (different amino acid sequences). This results in apparently great differences also in parts of the protein, where amino acid sequences are almost identical. Similarly few different amino acids in EF-Tu of *M. smegmatis* can induce significantly different conformation of the whole molecule.

4.2. Possible role of EF-Tu in cell signalling and regulation of the cell differentiation

4.2.1. Preparation of tools and building of experimental strategy

4.2.1.1. Protein sequence comparison of EF-Tu

Alignment of EF-Tu sequences from *Streptomyces* revealed very high degree of conservation of amino acid sequence (93-98 %), (Table 5). Noticeably, the residues in the GDP binding pocket (double underlined) and the conserved Thr_{385} position are identical. This Thr_{385}, corresponding to the phosphorylated Thr_{382} in *E. coli,* is absolutely conserved in all strains. Except for a few differences, the primary sequence of these proteins from *Streptomyces* is the same and so it is possible to assume that basic functional characteristics will be identical. It is therefore possible to use EF-Tu purified from *S. aureofaciens* in an *in vitro* kinase reactions with subcellular fractions (e.g. membrane fraction) from different *Streptomyces* strains as a protein kinase source to investigate the role of protein kinases in the regulation of differentiation.

```
Sau MAKAKFERTK PHVNIGTIGH IDHGKTTLTA AITKVLHDAY PEINPFTPFD 50
Sce ////////// ////////// ////////// ////////// /D//EASA//
Msm ////////// ////////// V/////////// ////////KF /DL/ESRA//

Sau QIDKAPEERQ RGITISIAHV EYQTEARHYA HVDCPGHADY IKNMITGAAQ 100
Sce ////////// ////////// ////////// //////////// //////////
Msm ///N////// /////N/S// ////DK//// ///A////// //////////

Sau MDGAILVVAA TDGPMPQTKE HVLLARQVGV PYIVVALNKA DMVDDEEILE 150
Sce ////////// ////////// ////////// ///////// //////////
Msm ////////// ////////R/ ////////// ///L/////S /A/////L//

Sau LVELEVRELL SEYEFPGDDL PVVRVSALKA LEGDKEWGEK LLGLMHAVDE 200
Sce ////////// /////////V ///K////// ////////NS V/E//K////
Msm ///M////// AAQD/DEEA/ ////////// ////PK/VKS VEE//E///A

Sau NIPTPARAVD QPFLMPIEDV FTITGRGTVV TGRIERGILK VNETVDIIGI 250
Sce A//E/E/D// K/////////// ////////// ///////V// //////////
Msm S//D/V/ET/ K/////VE// ////////// ///V///VIN ///E/E/V//

Sau KTEKTTTTVT GIEMFRKLLD EGQAGENVGL LLRGIKREDV ERGQVIIKPG 300
Sce ////////// ////////// ////////// ////////// //////////
Msm RP/T/K//// /V//////// Q////D//// ////////// /////VV///

Sau SVTPHTDFEA QAYILSKDEG GRHTPFFNNY RPQFYFRTTD VTGVVTLPEG 350
Sce //////E/// ////////// ////////// ////////// //////////
Msm TT////E//G QV/////////// ////////// ////////// //////////

Sau TEMVMPGDNT AMTVALIQPV AMEEGLKFAI REGGRTVGAG QVTKIVK 397
Sce ////////// E/K/E///// ////////// /////////// /////N/
Msm ////////// DIS/K///// //D///R/// /////////// R/T//I/
```

	Identity
Sau/Sce	**95 %**
Sau/Msm	**83 %**
Sce/Msm	**83 %**

Table 5. Sequence alignment of *tuf*1 deduced amino acid sequences from two *Streptomyces* species and *Mycobacterium smegmatis*. Abbreviations: Sau – *S. aureofaciens*, Sce – *S. coelicolor*, Msm - *Mycobacterium smegmatis*. The residues in the GDP binding pocket are marked in blue and double underlined and the conserved Thr_{385} position corresponding to the phosphorylated Thr_{382} in *E. coli,* is marked in red.

In order to unravel the role of EF-Tu in the process of bacterial differentiation we compared some of the properties of *Streptomyces* EF-Tu as a representative of differentiating *Actinomyces* with those of non-differentiating *Mycobacterium smegmatis*

(Table 5). *M. smegmatis* EF-Tu sequence was extracted from the partial genome sequence presented on "Unfinished microbial genomes database" at "The institute for genomic research" (TIGR - http://www.tigr.org) web page using "Blast" search program. There was determined a reasonably high degree of amino acid sequence identity (86.4 %) also between *S. aureofaciens* and *M. smegmatis* EF-Tu.

4.2.1.2. Cell cycle of *Streptomyces coelicolor* and *Mycobacterium smegmatis*

Posttranslational modifications of EF-Tu were investigated in two Actinomycetes: *Streptomyces coelicolor* and *Mycobacterium smegmatis*.

S. coelicolor is a soil differentiating bacterium, which sporulates. Scanning electron microscopy was used to analyse morphological differentiation of Streptomycetes on glass beads (Fig. 23). In this cultivation system mimicking natural environment of *Streptomyces* developed in our laboratory (Nguyen *et al.* 2005) were investigated and compared posttranslational modifications of EF-Tu during the differentiation.

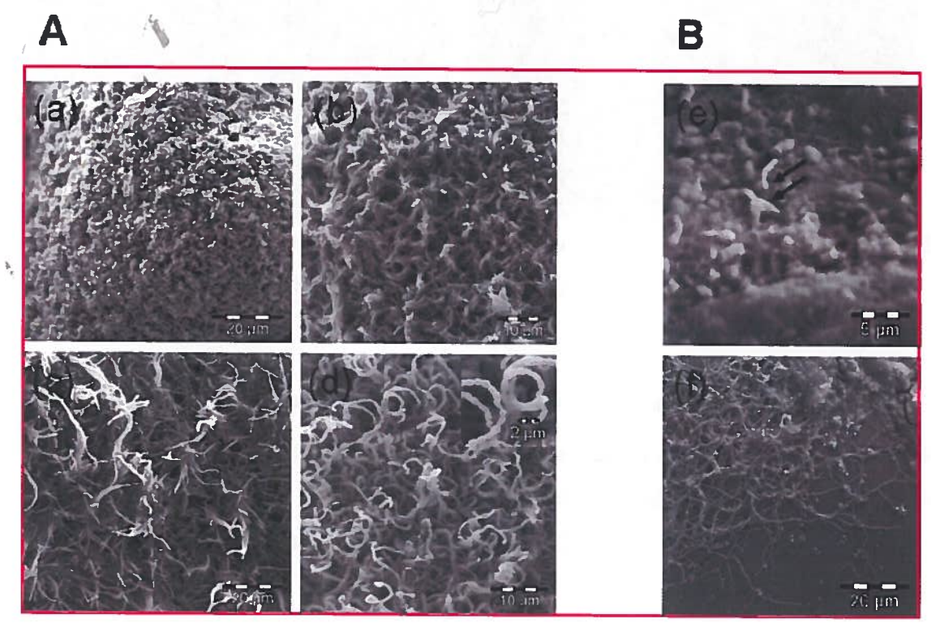


Figure 23. Morphological differentiation of *S. coelicolor* A3(2) grown on glass beads. In panel A, Figure (a) shows a young colony composed of substrate mycelia only. Later on, aerial mycelia started to stand up into the air (b) and elongate (c), this coincides with the

appearance of pigments. Later, coiled aerial mycelia start to form septa and spores (d). Images (a), (b), (c) and (d) were taken at 6, 16, 19 and 36 hours after transfer to glass beads, respectively. The magnified image in (d) shows an aerial mycelium septated to form spores. Panel B shows spores germinating on glass beads after 8 hours (e) and primary network of mycelium formed after 24 hours (f). One or two germ tubes coming from the spore were observed (arrows).

On the other hand *Mycobacterium smegmatis* is non-differentiating Actinomycetes, which does not sporulate. Here were analysed and compared posttranslational modifications of EF-Tu in exponential (22 hours of cultivation) and stationary (42 hours of cultivation) phase. Both cultures look in scanning electron microscope same (Fig. 24).

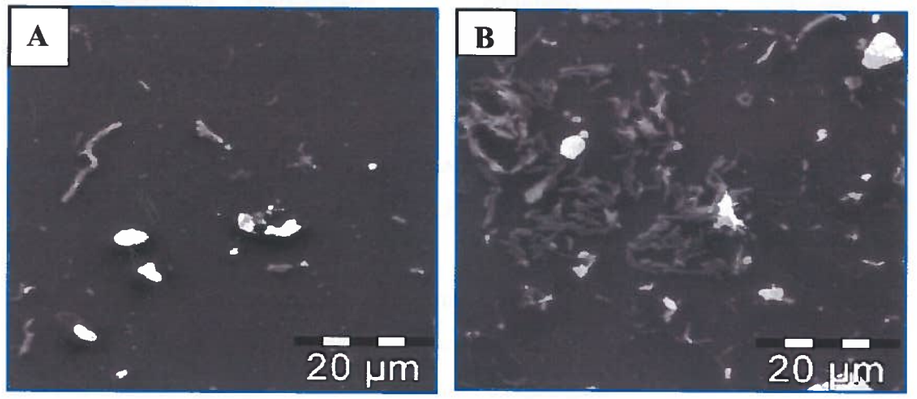


Figure 24. *Mycobacterium smegmatis* grown in liquid medium into: (A) exponential (22 h) and (B) stationary phase (42 h) observed under scanning electron microscope.

4.2.1.3. Presenceof EF-Tu in different strains and cell fractions

In order to secure identification of EF-Tu our polyclonal antibody raised against EF-Tu from *S. aureofaciens* was tested on different subcellular fractions from different bacterial strains and EF-Tu was detected in all of them (Fig. 25).

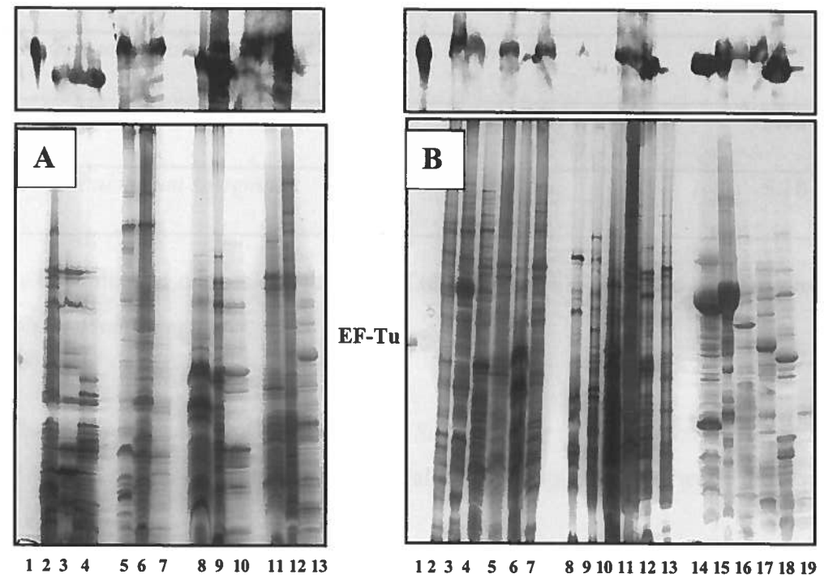


Figure 25. A: Membrane, ribosome and S150 fractions numbered respectively, from the following sources: *E. coli* (2 – 4), *Mycobacterium smegmatis* (5—7), *S. aureofaciens* 84/25 (8—10) and *S. coelicolor* (11—13), B: Samples from *S. aureofaciens* 3239, *S. granaticolor*, *S. collinus*, *S. coelicolor*, *S. aureofaciens* 84/25, *S. lividans* respectively representing membrane (2—7), ribosomal (8—13) and S150 (14—19) fraction. (1) – standard of EF-Tu. EF-Tu in all tested fractions was detected by Western blot technique. The result is shown in panels above the silver stained gels.

Although EF-Tus from different bacterial strains have high degree of amino acid sequence identity, they differ in their pI values (Table 6). This fact is quite important for identification of EF-Tu.

Bacterium	Molecular mass (Da)	Theoretical pI
Streptomyces aureofaciens	**43,683.00**	**5.21**
Streptomyces coelicolor	**43,780.94**	**5.01**
Mycobacterium smegmatis	**43,703.66**	**5.18**

Table 6. Molecular masses and theoretical pI values of EF-Tus from two *Streptomyces* and *Mycobacterium smegmatis*.

The difference of 0.2 pH units in pI of *Streptomyces* (*S. aureofaciens* and *S. coelicolor*) and *Mycobacterium smegmatis* (Table 6) allows distinguishing between them on 2D-gels quite easily.

4.2.2. Presence and heterogeneity of EF-Tu in differentiating and non-differentiating *Actinomyces*

The two-dimensional gel electrophoresis provides an extremely powerful and widely used technique for separating proteins in cell extracts. Since its development, this technique has been adopted for use in several ways. In particular, it has been developed for the investigation of covalent modifications of proteins, namely for the monitoring of changes in phosphorylation state of proteins (Idelson and Amster-Choder 1998; Maurides *et al.* 1989). Using combination of 2D-PAGE and Western blot, charge heterogeneity of EF-Tu was analysed in the subcellular fractions of *Streptomyces coelicolor* and *Mycobacterium smegmatis*. pI of the gels was 4 – 7 in all of our experiments. EF-Tu was found not only in the cell soluble fraction but also on membranes and moreover, the pattern of isoforms of the factor in these fractions was different in *S. coelicolor* (Fig. 26) and *M. smegmatis* (Fig. 27). These patterns are probably the result of posttranslational modifications of EF-Tu and they depend on the cell localisation of this protein and developmental stage of bacterial culture.

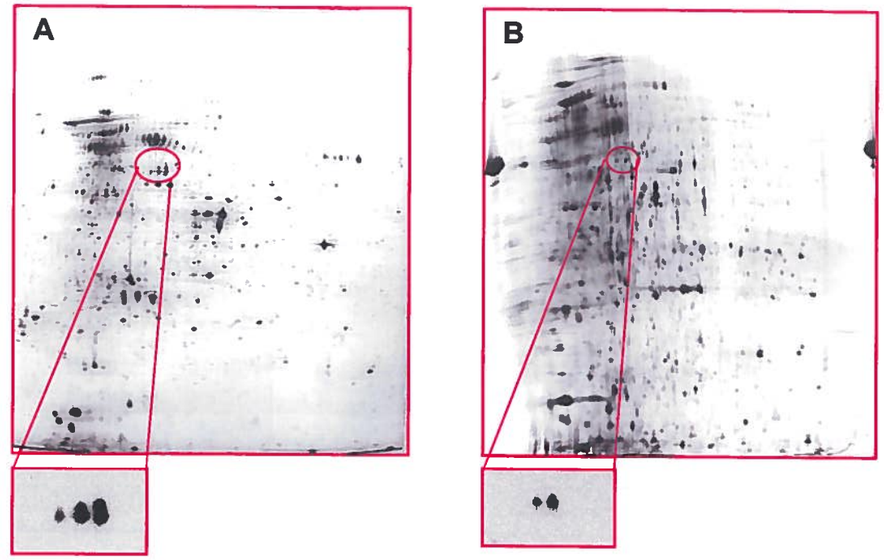


Figure 26. Comparison of heterogeneity of EF-Tu population in the membrane (A) and soluble S30 fraction (B) in aerial mycelium of *Streptomyces coelicolor* grown on glass beads for 19h. Blow up of EF-Tu region on Western blot is below the silver stained gels.

In the membrane fraction of non-differentiating *Mycobacterium smegmatis* EF-Tu was found in two isoforms, while in cell soluble S30 fraction it was found to be represented by three isoforms (Fig. 27).

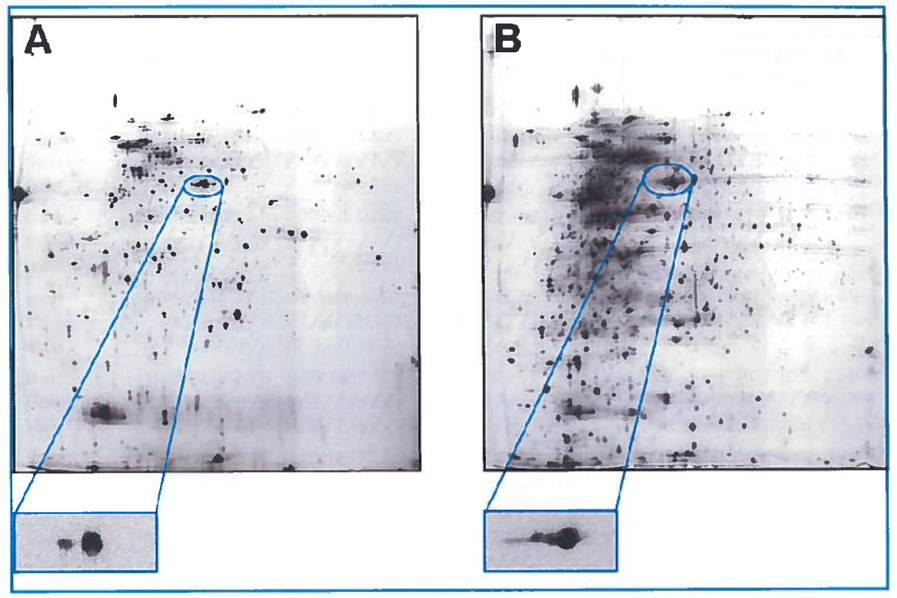


Figure 27. Comparison of heterogeneity of EF-Tu population in the membrane (A) and soluble S30 fraction (B) in *Mycobacterium smegmatis* grown in liquid medium. Blow up of EF-Tu region on Western blot is below the silver stained gels.

4.2.3. Phosphorylation of EF-Tu during growth and differentiation of *Actinomyces*

One of the well described posttranslational modifications of EF-Tu is phosphorylation (Lippmann *et al.* 1993). In *Streptomyces* we and our colleagues have shown using monoclonal antibodies against phospho amino acids that EF-Tu is phosphorylated on threonine and serine residues (Mikulik and Zhulanova 1995), (Nguyen *et al.* in press). One of the likely sites is the threonine 385, equivalent to Thr_{382} in *E. coli*, which was identified to be phosphorylated by Lippmann (Lippmann *et al.* 1993). Table 7 shows all potential Thr phosphorylation sites of EF-Tus from three *Actinomyces,* which are unique for *Streptomyces* (red) or *Mycobacterium* (green).

```
Sau     MAKAKFERTK PHVNIGTIGH IDHGKTTLTA AITKVLHDKY PEINPFTPFD 50
Scoe    ////////// ////////// ////////// ////////// ////E/T/// 
Msm     ////////// ////////// V/////////// ////////F ////ESRA// 

Sau     QIDKAPEERQ RGITISIAHV EYQTEARHYA HVDCPGHADY IKNMITGAAQ 100
Scoe    ////////// ////////// ////////// ////////// ////////// 
Msm     ///N////// /////N/S// ////DK//// ///A////// ////////// 

Sau     MDGAILVVAA TDGPMPQTKE HVLLARQVGV PYIVVALNKA DMVDDEEILE 150
Scoe    ////////// ////////// ////////// ////////// ////////// 
Msm     ////////// ////////R/ ////////// ///L////S /A////L// 

Sau     LVELEVRELL SEYEFPGDDL PVVRVSALKA LEGDKEWGEK LLGLMHAVDE 200
Scoe    ////////// /////////V ///K////// ////////NS V/E//K//// 
Msm     //M////// AAQ//DEEA- ////////// ////PK/VKS VEE//E///A 

Sau     NIPTPARAVD QPFLMPIEDV FTITGRGTVV TGRIERGVLK VNETVDIIGI 250
Scoe    A//E/E/D// K/////////  ////////// ////////// ///T////// 
Msm     S//D/V/E// ////////// ////////// ///V////IN ///E/E/V// 

Sau     FTEKTTTVT GIEMFRKLLD EGQAGENVGL LLRGIKREDV ERGQVIIKPG 300
Scoe    /T///T//// ////////// ////////// ////////// ////////// 
Msm     RP/T/K//// /V/////////  Q////D//// ////////// /////VV/// 

Sau     SVTPHTDFEA QAYILSKDEG GRHTPFFNNY RPQFYFRTTD VTGVVTLPEG 350
Scoe    //////E/// ////////// ////////// ////////// ////////// 
Msm     TT//////G  QV/////////  ////////// ////////// ////////// 

Sau     TEMVMPGDNT AMTVALIQPV AMEEGLKFAI REGGRTVGAG QVTKIVK    397
Scoe    ////////// E/K/E///// ////////// ////T///// /////N/ 
Msm     ////////// /IS/K///// //D///R/// /////T///// R////I/ 
```

Table 7. *S. aureofaciens* (Sau), *S. coelicolor* (Scoe) and *M. smegmatis* (Msm) EF-Tu sequence alignment with marked threonine residues unique for *Streptomyces* (red) or *Mycobacterium* (green). Threonine 385 equivalent to Thr_{382} in *E. coli* is marked in orange. Sequences were obtained from www.expasy.org.

Since it is important to distinguish between own EF-Tu from *S. coelicolor* and externally added factor from *S. aureofaciens* on 2D gel, we first localized their position in our 2D electrophoresis system by Western blotting (Fig. 28).

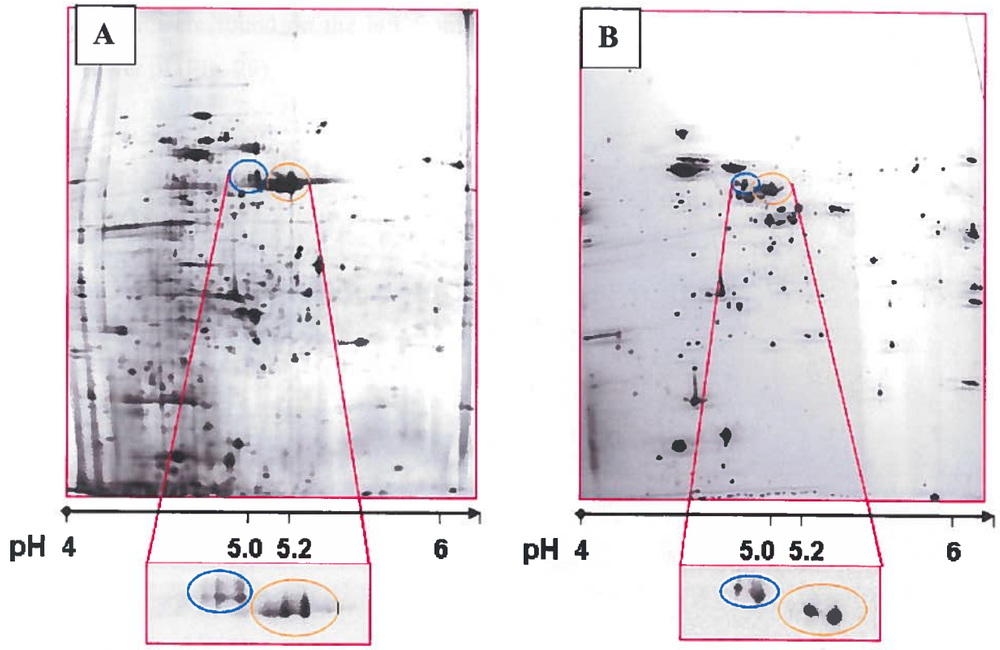


Figure 28. Detection of own (blue) and externally added purified EF-Tu from *S. aureofaciens* (orange) in membrane fraction of *S. coelicolor* cultivated 6 hours on glass beads (A) and in liquid medium for 20 hours (B) by 2D electrophoresis. EF-Tu detected by Western blot is below silver stained gel.

4.2.3.1. EF-Tu in spores

Elongation factor Tu is present also in dormant spores (Weiser *et al.* 1989). Its presence was proved in crude cell extract by Western blot (results not shown). There were always detected fragments of the factor as a result of intense proteolytic activity during protein sample preparation. We were not able to inactivate them even if we used number of different protease inhibitors.

Crude spore cell extract was found to contain protein kinase(s), which phosphorylated externally added EF-Tu from *S. aureofaciens* (Fig. 29). We found two phosphorylated isoforms and phosphate group(s) were transferred not only on externally added *S. aureofaciens* purified factor, but also on that of *S. coelicolor* host. Spots representing host

factor were found on the left from spots of *S. aureofaciens* EF-Tu corresponding to its lower pI (Fig. 29).

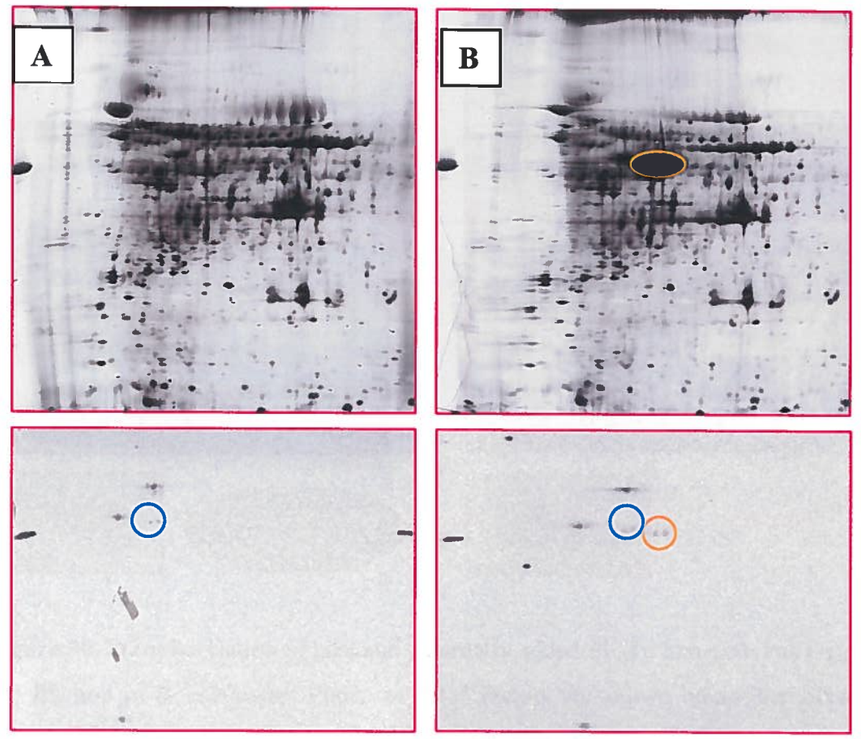


Figure 29. Host and externally added EF-Tu phosphorylation analyzed by 2D gel electrophoresis in crude cell extracts (CE) from spores of *S. coelicolor*. (A) CE without added EF-Tu, (B) CE with added *S. aureofaciens* EF-Tu circled in orange, own EF-Tu is circled in blue. Phospho proteome images are below silver stained gels.

4.2.3.2. Phosphorylation of EF-Tu in *S. coelicolor*

To see whether phosphorylation of EF-Tu depends on its localisation in the cell, we analysed phospho proteomes of membranes and S30 fractions prepared by *in vitro* kinase reactions, where the above mentioned fractions were the source of kinases.

S. coelicolor EF-Tu as well as added *S. aureofaciens* factor were found to be phosphorylated in both fractions (Fig. 30), however, number and concentration of

phosphorylated isoforms was different. This suggests that phosphorylation of EF-Tu can play a modified role in different cell compartments.

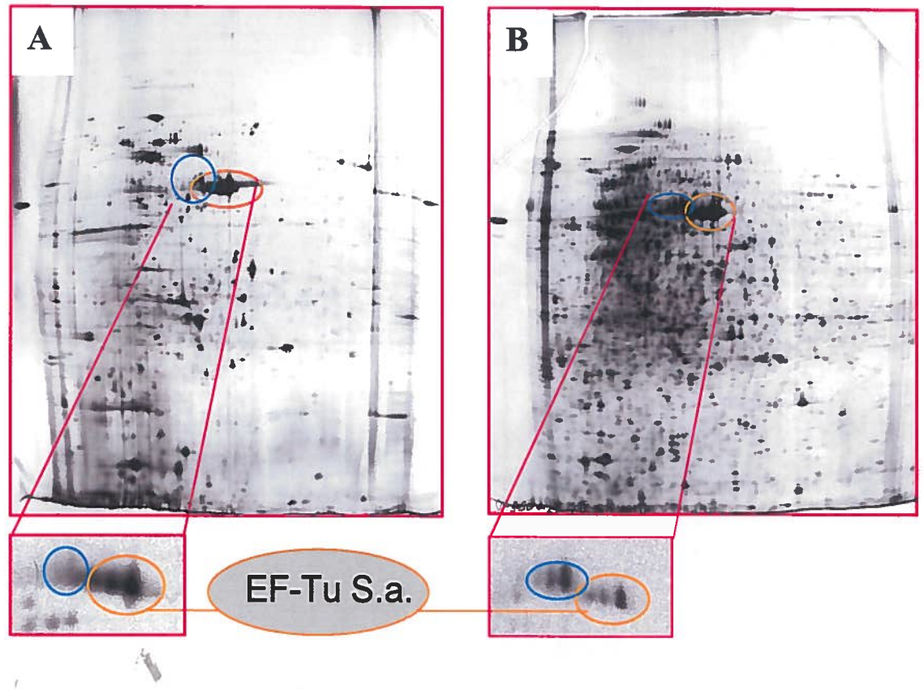


Figure 30. Phosphorylation of host and externally added EF-Tu in membrane (A), and S30 (B) fraction of *S. coelicolor*. Phosphorylated factors are shown below the silver stained gels. Fractions are from culture grown 6 h on glass beads. Externally added EF-Tu is circled in orange, host EF-Tu in blue.

4.2.4. Attempt to identify specific protein kinases phosphorylating EF-Tu

Since we were interested in the kinase(s), which are responsible for EF-Tu phosphorylation in *Streptomyces*, we analysed membrane phospho proteome of wild type *S. granaticolor* and compared it with that of *Δpkg2* mutant *S. granaticolor* (kindly provided by Dr. P. Branny), which possessed inactivated PKG2 kinase. PKG2 kinase is threonine/serine type of membrane protein kinase (Nadvornik *et al.* 1999).

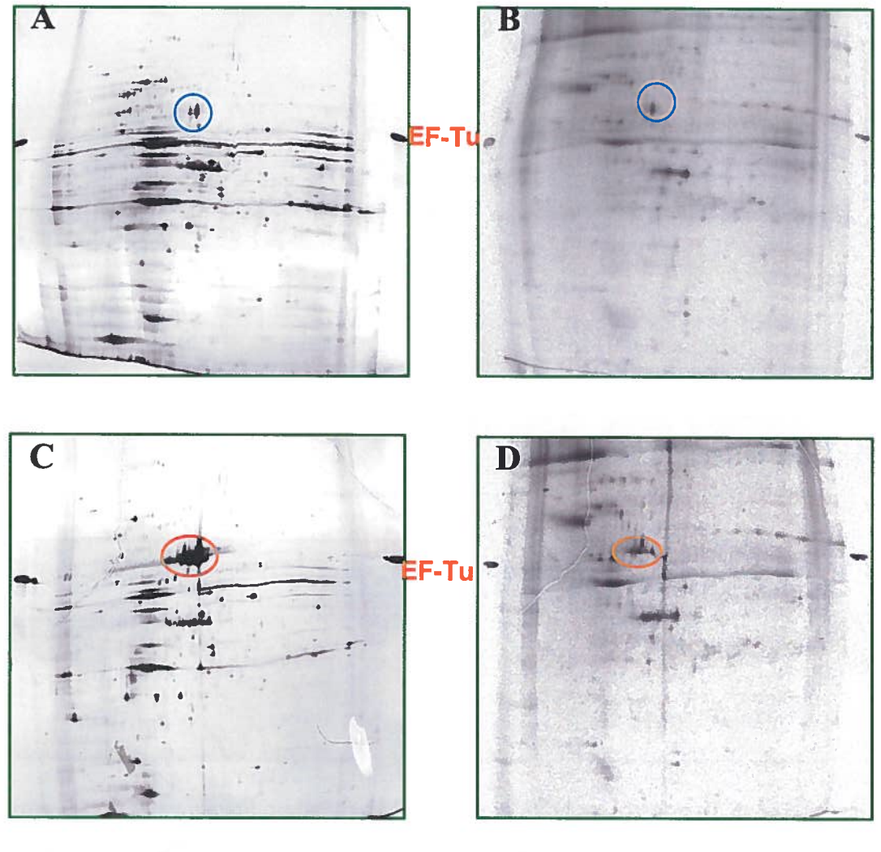


Figure 31. *In vitro* phosphorylation of EF-Tu in membrane fraction of *Δpkg2* mutant of *Streptomyces granaticolor*. The culture was grown in liquid medium for 14 – 16 hours (young culture). (A, C) silver stained gels, (B, D) phospho images. In C and D there was externally added EF-Tu from *S. aureofaciens*, which is circled in orange. Host EF-Tu is circled in blue.

We observed that phosphorylation of EF-Tu in membrane fraction from *Streptomyces granaticolor* depends on the age of the culture. It is phosphorylated in the sample from the young culture (before production of granaticin starts), (Fig. 31), but it is not in the old one (producing granaticin), (Fig. 32). We also showed that PKG2 kinase is not responsible for phosphorylation of EF -Tu, since it is clearly phosphorylated in deletion mutant (*S. granaticolor Δpkg*2) as well as in the wild type (Figs. 31 and 32).

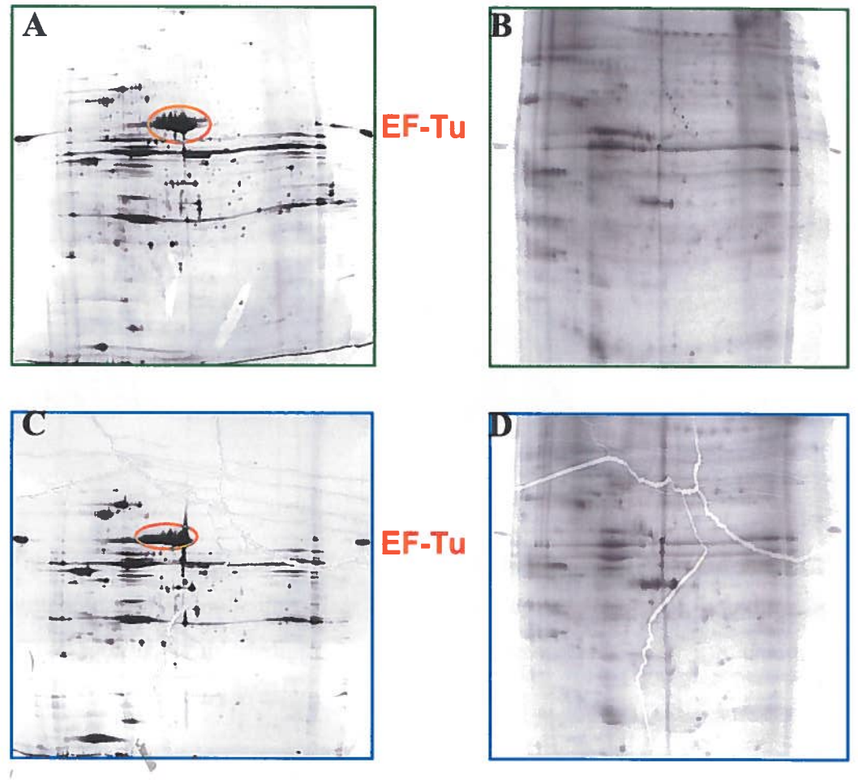


Figure 32. *In vitro* phosphorylation of EF-Tu in membrane fraction of *Δpkg2 Streptomyces granaticolor* mutant (A and B) and in the wild type (C and D). The culture was grown in liquid medium for 24 hours (old culture). (A, C) silver stained gels, (B, D) phospho images. Externally added EF-Tu from *S. aureofaciens* is circled in orange.

4.2.5. Heterogeneity and phosphorylation of EF-Tu present in the membrane proteome of differentiating and non-differentiating *Actinomyces*

It was shown that elongation factor Tu is present in membranes of *Streptomyces* and other bacteria (Weiser *et al.* 1989; Granato *et al.* 2004; Dallo *et al.* 2002). Its isoform profile differs depending on a bacterial strain, cultivation conditions and developmental stage (Fig. 33). This indicates that posttranslational modifications of this protein could have some function specific for a given strain.

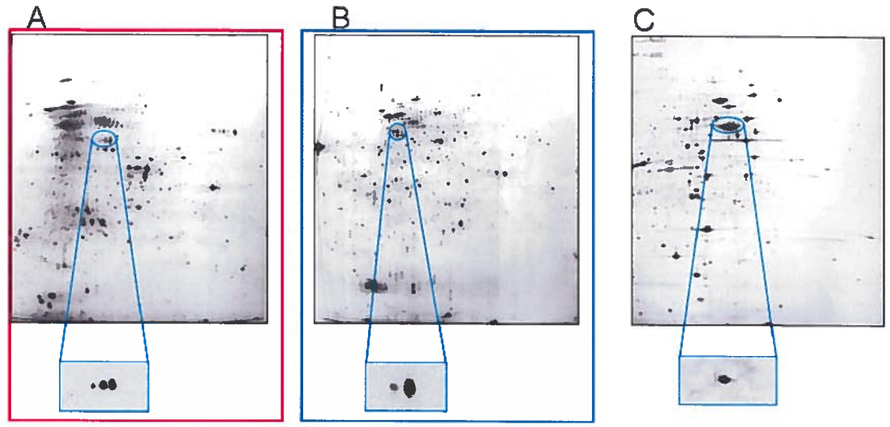


Figure 33. Presence and heterogeneity of EF-Tu population in bacterial membrane proteome. (A) *S. coelicolor*, glass beads 19 h, (B) *M. smegmatis*, liquid medium, 22 h and (C) *B. subtilis*, liquid medium, 20 h. Silver stained gels are at the top, charge heterogeneity of EF-Tu detected by Western blot technique is below.

4.2.5.1. Membrane localisation of EF-Tu

To prove embedding of EF-Tu in membranes we repeatedly centrifuged membrane fraction through 30% sucrose gradient at 20,000 r.p.m. in SS-34 rotor for 1 hour. EF-Tu survived this treatment bound to the membranes (Fig. 34 – (2)). Moreover, after treatment with 1M ammonium chloride for 1 hour and centrifugation, EF-Tu was still present in membrane fraction (Fig. 34 – (3)).

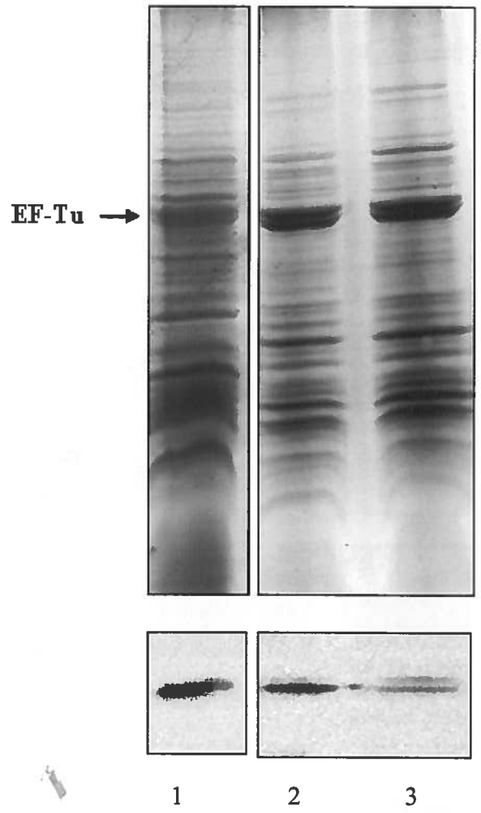


Figure 34. SDS electrophoresis analysis of stability of EF-Tu binding to membranes after their purification. 30 μg of crude membrane fraction (1), membranes purified by centrifugation through sucrose cushion (2), purified membranes treated by 1M ammonium chloride (3). EF-Tu detected by polyclonal antibody is shown on Western blots below Coomassie blue stained gel.

4.2.5.2. Membrane proteome

The posttranslational modifications play a key role in cell signalling, therefore we analysed EF-Tu heterogeneity in membrane proteome of *S. coelicolor* during morphological differentiation by 2D electrophoresis and compared it with that of non-differentiating Actinomycetes *M. smegmatis*.

In *S. coelicolor* were analysed and compared membrane samples from vegetative mycelium (6 h of cultivation on a glass beads), aerial mycelium (19 h of cultivation on a glass beads) and from the early stage of sporulation (34 h of cultivation on a glass beads),

(Fig. 35). These morphological stages of differentiation were documented by electron microscopy (Fig. 23), (Nguyen *et al.* 2005).

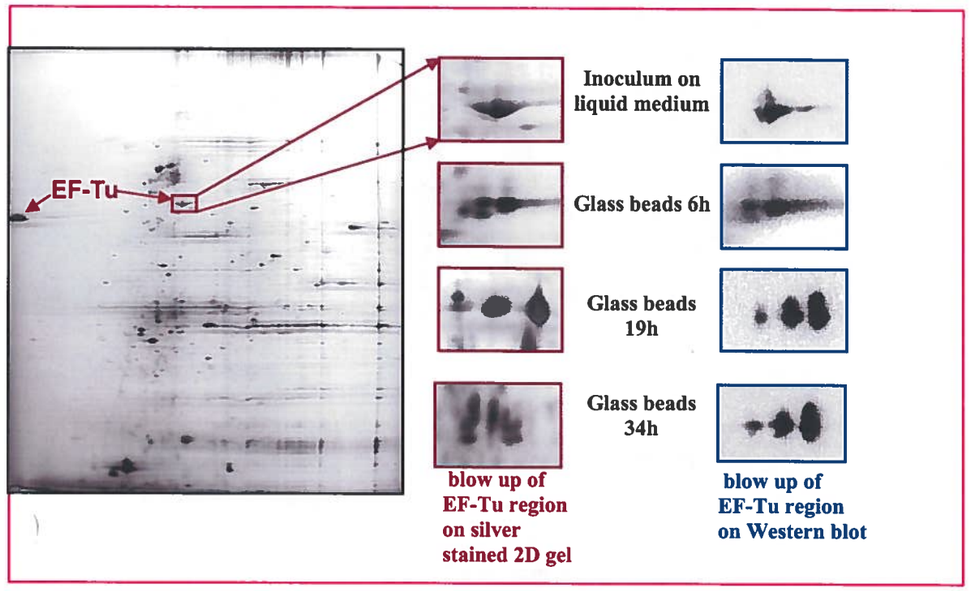


Figure 35. Analysis of EF-Tu heterogeneity in membrane proteome of *Streptomyces coelicolor* during morphological differentiation on glass beads. Whole silver stained gel is on the left. Zoomed out regions where EF-Tu is localised and their Western blots are on the right.

Western blot analysis of EF-Tu present in the membrane fraction of *S. coelicolor* in the course of morphological differentiation supported by the growth on glass beads revealed quite extensive charge heterogeneity of factor isoforms. Two isoforms of EF-Tu were found in membrane fraction from vegetative mycelium (6 h), three isoforms in membrane fraction from aerial mycelium (19 h), and also three isoforms in the early stage of sporulation (34 h). These results show that EF-Tu localized on membranes is probably posttranslationally modified and these modifications change during the morphological differentiation.

In *M. smegmatis* were analysed and compared membrane fractions from liquid culture grown into exponential and stationary phase (Fig. 36).

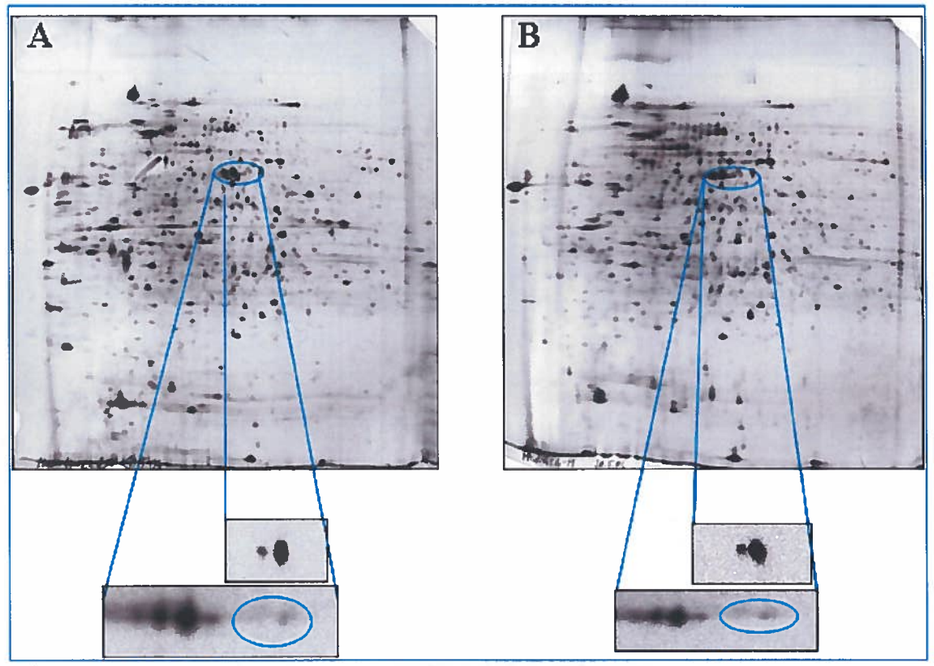


Figure 36. Membrane fraction of *Mycobacterium smegmatis* grown in a liquid medium into (A) exponential phase (22 h of cultivation) and (B) stationary phase (42 h of cultivation). Silver stained gels are at the top, charge heterogeneity of EF-Tu detected by Western blot technique is in the middle and at the bottom are phosphorylated proteins. Phosphorylated EF-Tu of *M. smegmatis* is circled in blue.

Two isoforms of EF-Tu were found and both of them were phosphorylated in an *in vitro* kinase reaction. Their distribution was not growth dependent.

4.2.5.3. Membrane phospho proteome of *S. coelicolor* during differentiation

Analysis of membrane phospho proteome of *S. coelicolor* during the course of morphological differentiation supported by the growth on glass beads revealed multiple phosphorylation of EF-Tu, which seems to be dependent on the stage of morphological differentiation (Fig. 37).

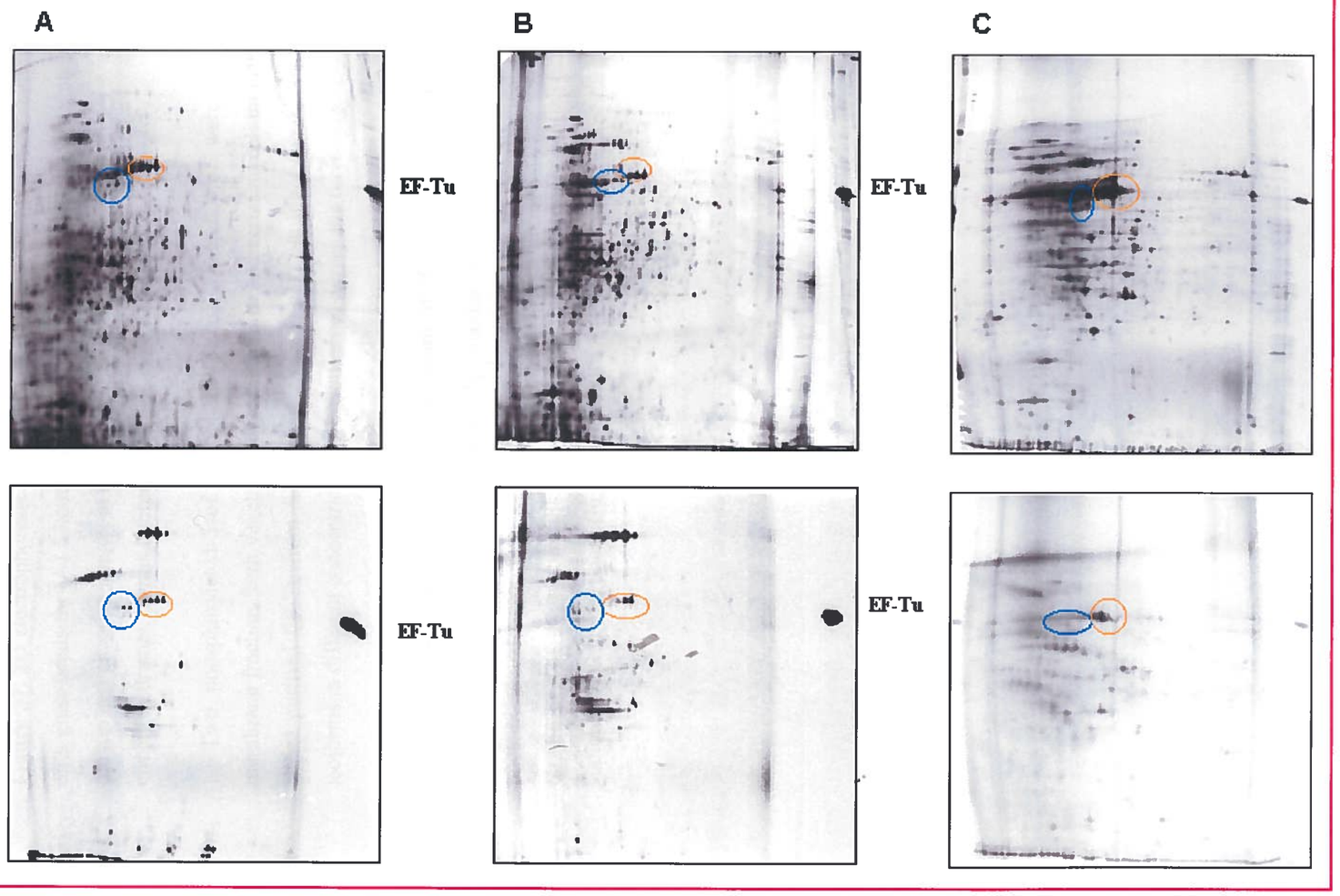

A
B
C
EF-Tu
EF-Tu
EF-Tu
EF-Tu
EF-Tu

Figure 37. 2D electrophoresis of membrane phospho proteome of *S. coelicolor* cultivated on a glass beads for (A) 6 hours, (B) 19 hours and (C) 34 hours. Silver stained gels are at the top and phospho images at the bottom. Externally added EF-Tu from *S. aureofaciens* is circled in orange, own is in blue.

Two phosphorylated isoforms of EF-Tu of *S. coelicolor* were identified in the membrane fraction from vegetative mycelium (6 h) and aerial mycelium (19 h). Externally added purified EF-Tu from *S. aureofaciens* was also phosphorylated, however, all three isoforms in different concentrations were observed during the three stages.

4.2.5.4. Membrane phospho proteome of *M. smegmatis* during the growth

Analysis of membrane phospho proteome of *M. smegmatis* revealed two phosphorylated isoforms of EF-Tu, whose concentration and position were not dependent on the age of the culture. Their pattern was the same in exponential (22 hours) as in stationary (42 hours) phase (Fig. 38 and 39).

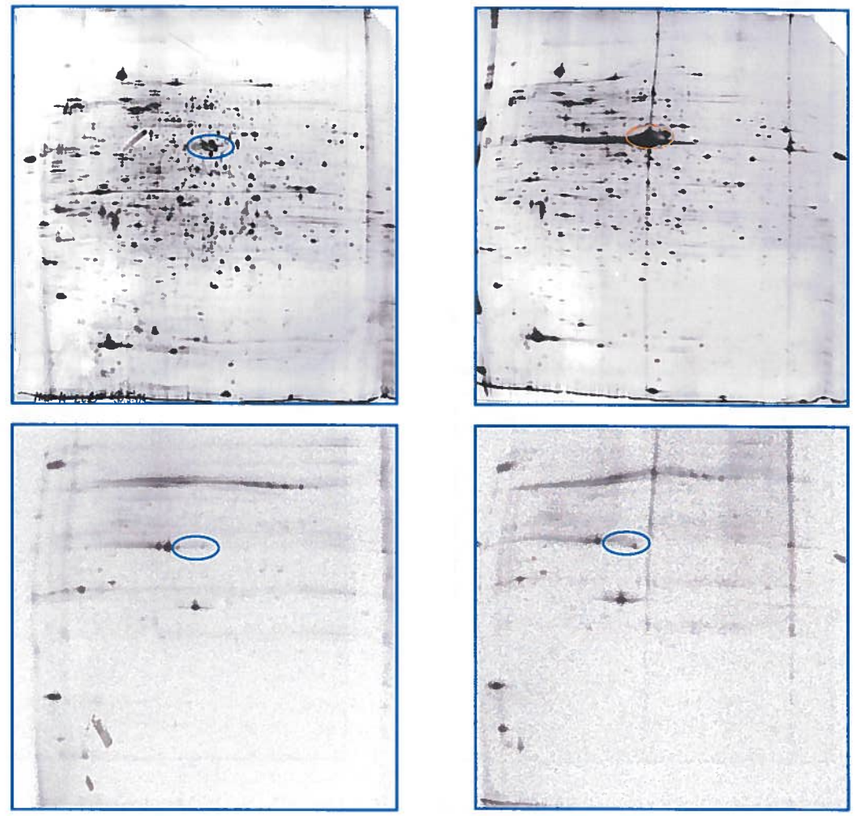


Figure 38. Phosphorylation of membrane proteins from *M. smegmatis* during 22 h growth (young culture). Silver stained gels are at the top and phospho images at the bottom. In the right half there are gels with samples where there was externally added EF-Tu from *S. aureofaciens*, which is circled in orange. EF-Tu of *M. smegmatis* is circled in blue.

We made very interesting observation in this experiment revealing that only own EF-Tu of *M. smegmatis* was phosphorylated while the phosphorylation of externally added purified EF-Tu from *S. aureofaciens* was not detected contrary to experiments with *Streptomyces* membrane proteomes.

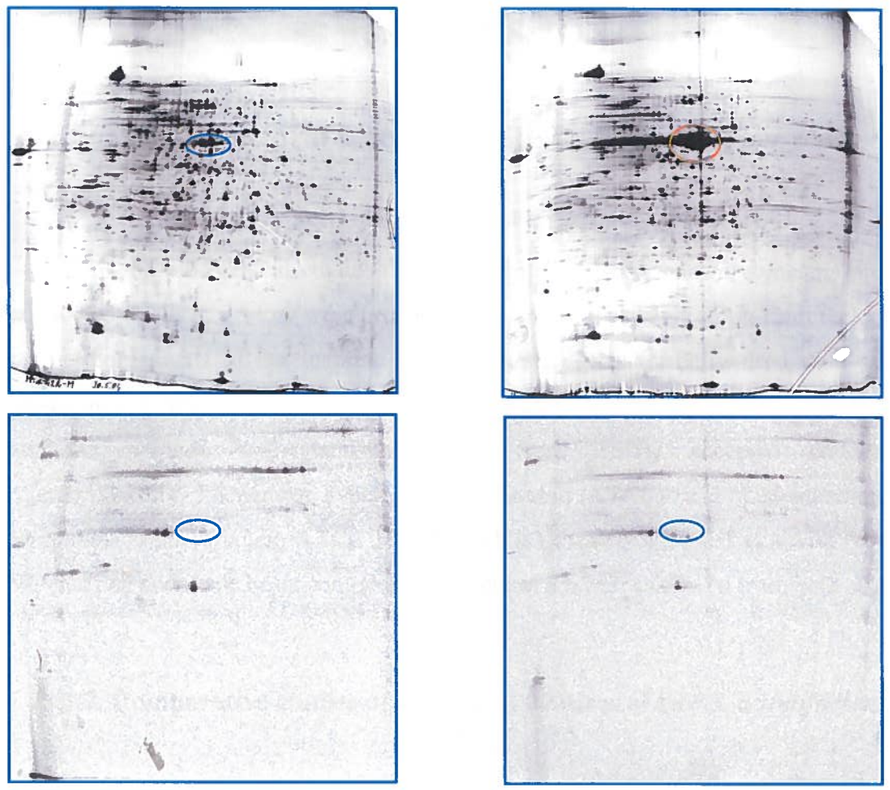


Figure 39. Phosphorylation of membrane proteins from *M. smegmatis* during 42 h growth (old culture). Silver stained gels are at the top and phospho images at the bottom. In the right half there are gels with samples where there was externally added EF-Tu from *S. aureofaciens*, which is circled in orange. EF-Tu of *M. smegmatis* is circled in blue.

5. DISCUSSION

5.1. Structural features of EF-Tu from *Streptomyces*

5.1.1. Historical background

All of the biochemical studies on EF-Tu from *Streptomyces aureofaciens* previously conducted in our laboratory were performed on strain 84/25. This strain from the collection of microorganisms of the Institute of Microbiology was formerly used as a strain for production of chlortetracyclines and his origin was quite unclear. Therefore we turned our attention towards well-established strain from widely accepted collection of microorganisms - "American Type Culture Collection (ATCC)" and obtained strain named *Streptomyces aureofaciens* ATCC 10762 which is generally accepted as a wild type strain. We purified and made basic comparative structural analysis of EF-Tu from both strains.

5.1.2. Comparative studies of structural features of two *S. aureofaciens* strains

During the purification procedure of two factors were observed few differences. We gained relatively greater ratio of EF-Tu to other proteins in fractions from wild type *S. aureofaciens* after ion-exchange chromatography. The protein from this strain also aggregated more readily than the factor from *S. aureofaciens* 84/25. During tryptic digestion and during incubation at 37 °C, EF-Tu from *S. aureofaciens* 10762 seemed less stable than that of *S. aureofaciens* 84/25, since more fragments and less intact EF-Tu was detected. These differences could be explained by physiological variance between the strains, nevertheless they led us to the analysis of the primary sequence of *S. aureofaciens* ATCC 10762 EF-Tu and its comparison with that of *S. aureofaciens* 84/25 (GeneBank accession no. AF007125).

5.1.3. Primary sequence analysis of EF-Tu from *S. aureofaciens* ATCC 10762

We sequenced *tuf*1 gene from the wild type *S. aureofaciens* 10762 using the method of chain termination (dideoxynucleotides with a fluorescent tag) and compared it with *tuf*1

gene of tetracycline producing strain *S. aureofaciens* 84/25. Although we observed number of differences in nucleotide sequence, the deduced amino acid sequences of both EF-Tus were absolutely identical. *S. aureofaciens* 84/25 strain passed probably many mutation processes during improvement of its antibiotic production potential, however those did not affect primary structure of the protein. Nonetheless obtained amino acid sequence was a result of "*in silico*" experiment made by computer using the program Chromas. In natural environment, differences in nucleotide sequence can lead to different properties of mRNA and some codon triplets could be misread leading to different amino acid sequence of real protein and thus influencing its properties.

We presented the alignment of the deduced amino acid sequences of four *Streptomyces* EF-Tu species. They showed very high identity levels, which range between 94 % and 98 % while those with *E. coli* or *M. smegmatis* factor are only about 72-75 %. This high identity of sequences among all *Streptomyces* EF-Tu(s), in principle, allowed us to use in our experiments different *Streptomyces* strains and generalize the findings for all the factors.

5.1.4. 3D comparative model structures of EF-Tu

Since there is quite high identity between amino acid sequences of EF-Tu(s) in bacteria, it is possible to use the model of EF-Tu from *E. coli*, which was crystallised and its 3D structure determined (Song *et al.* 1999), as a template and construct the 3D structure model of EF-Tu from Streptomycetes or Mycobacteria. We used Deep-View/Swiss-PbdViewer program for that purpose (Peitsch 1995; Guex and Peitsch 1997; Schwede *et al.* 2003).

Models of EF-Tu from *S. aureofaciens* and *S. coelicolor* are almost identical. It is a consequence of almost 100% identity of their amino acid sequences. Although the differences between the primary structure of Streptomycetes EF-Tu and that of *M. smegmatis* are somewhat more extensive (around 86 % of identity in amino acid sequence), it still allows to build a reliable 3D model of Mycobacterium EF-Tu and to make the comparisons. The comparison of EF-Tu 3D structures from *Streptomyces* and *Mycobacterium* revealed very similar overall shape of the molecule, however the orientation of domains differs significantly, which results in the presence of a "tunnel like space" in the molecule of EF-Tu with different shape in *Streptomyces* than in *M. smegmatis*

EF-Tu. This "tunnel" is made by switch (inter domain) regions, which change significantly upon GTP binding (Song *et al.* 1999).

One of the characteristic features of EF-Tu is the ability of the protein to aggregate, however the conditions required for aggregation differ in different bacteria. In case of *S. aureofaciens* this aggregation is spontaneous under physiological conditions and enables rapid purification of this protein (Weiser *et al.* 1982). On the other hand, EF-Tu from *S. collinus* aggregates only if it is purified and EF-Tu from *S. coelicolor* does aggregate neither in extract nor purified in physiological conditions mimicking buffers. Responsible for the aggregation are usually hydrophobic amino acids, which are on the surface of the protein. Only single change in one surface amino acid from polar to hydrophobic can make the whole protein hydrophobic and capable of aggregation. For this reason we were searching in our models for hydrophobic amino acids in *S. aureofaciens*, which substitute some of polar amino acids in *S. coelicolor*. Contrary to *E. coli*, we did not find any. For spontaneous aggregation of the factor from *S. aureofaciens* is (are) probably responsible some amino acid(s), which differ(s) from EF-Tu of *S. coelicolor*, even though their charge is similar. Most probably it is one or more from the "red" region (Table 4), since there is number of different amino acids in a relatively small region.

5.1.5. 2D analysis of heterogeneity of purified EF-Tu

2D electrophoresis of purified elongation factor Tu revealed at least three isoforms of this protein, which were in number of control experiments, including over-expression of the protein in *E. coli* (Nguyen *et al.* in press), excluded to be an artefacts. This means that this protein is posttranslationally modified and these modifications, which confer factor different charge, are most probably phosphorylations since the isoforms reacted with monoclonal antibodies against phosphothreonine and phosphoserine. Nevertheless, also other modifications can play a role in charge heterogeneity of this protein and phosphorylation might be a secondary effect. To distinguish between the two possibilities it is necessary to determine the type and location of posttranslational modification(s) with the help of mass spectrometry. These studies are under way in the Laboratory of characterisation of molecular structures in the Institute of Microbiology.

5.2. Posttranslational modifications of EF-Tu and its potential role in the cell signalling

The strategy in the studies of posttranslational modifications of EF-Tu in *Streptomyces* and its possible role in the cell signalling and regulation of differentiation was based on a very high degree of amino acid sequence identity between *Actinomyces* EF-Tus and thus their interchangeability, easiness of purification of EF-Tu from *S. aureofaciens* and comparison of data from differentiating *Streptomyces* and non-differentiating *Mycobacterium smegmatis*.

We raised polyclonal antibody against elongation factor Tu of *S. aureofaciens* in rabbits. After purification from their serum, this antibody was shown to react specifically with EF-Tu. Moreover this antibody detected not only EF-Tu from Streptomycetes, but also that of *M. smegmatis*, *Bacillus subtilis* and *E. coli* as a representative of Gram-negative bacteria.

EF-Tu in many bacteria was found not only in cell soluble and ribosomal fractions, where it would be expected, but it was also shown to be associated to the periplasm of *Neisseria gonorhoeae* (Porcella *et al.* 1996), to the cell wall of *Mycobacterium leprae* (Marques *et al.* 1998) and to the membranes in *Streptomyces* (Weiser *et al.* 1989), (Kim *et al.* 2005). Here it might have additional important biological functions. It could for instance act as an adhesine molecule (Granato *et al.* 2004) or it could be a convenient supplement of regulations by ppGpp since in *E. coli*, *Bacillus subtilis* and *Bacillus licheniformis*, a part of EF-Tu population that is located on the membrane can be methylated in response to starvation for an essential nutrient (Young *et al.* 1990; Young and Bernlohr 1991).

We proved by centrifugation through sucrose cushion and ammonium chloride treatment that EF-Tu of *S. coelicolor* is bound to the membrane.

By combination of 2D-PAGE and Western blotting, the presence of EF-Tu was analysed in the subcellular fractions of *Streptomyces coelicolor* and *Mycobacterium smegmatis*. This revealed extensive heterogeneity of the factor. Its isoforms were distributed differently depending on EF-Tu localisation. This means that the protein might play some other role than that in protein synthesis. When localised on membranes it could have for instance some function in a cell signalling and communication between cell environment and its translation system.

Since our preliminary studies showed that the charge heterogeneity of the factor is dependent on the time of growth in a liquid medium (Nguyen 2001), we have focused our further experiments on analysis of heterogeneity of the protein when cells undergo morphological differentiation on solid surface of glass beads. This system offers an alternative for studies of morphological and biochemical differentiation under conditions more similar to those in nature. The system not only mimics surface dehydration of soil but also provides mycelial growth and developmental compartments that include deeper regions where mycelia are submerged in a liquid medium. This creates environmental-like conditions for signalling in the population. We were comparing heterogeneity of EF-Tu in two Actinomycetes, differentiating and sporulating *S. coelicolor* and that of non-differentiating *M. smegmatis*. We focused on the membrane fraction since it is a point of entry in the cell signalling.

Western blot analysis of EF-Tu present in membrane fraction of *S. coelicolor* in the course of morphological differentiation supported by growth on the glass beads revealed quite extensive charge heterogeneity of the factor which might be due to its posttranslational modification(s). They might represent phosphorylations or other yet unknown posttranslational modification of EF-Tu regulating its function related to the cell differentiation, intracellular or extracellular signalling or other functions in the cell. EF-Tu is probably incorporated in a membrane in such a way that it can accept and transmit signals.

EF-Tu was detected by Western blot also in dormant spores of *S. coelicolor*, however, the major part of its population was digested during sample preparation. This was probably caused by EF-Tu non-specific degradation by proteases, which are extremely active at this stage of morphological development and we failed to inactivate them even if using almost all the protease inhibitors available (Protease Inhibitors Set, Roche and Protease Inhibitor Cocktail, Sigma) in maximum concentrations during harvesting and fractionation of bacteria. Proteolysis of EF-Tu was also observed in other stages of *S. coelicolor* life cycle and was most marked in samples from *in vitro* phosphorylation reactions, where the factor is exposed to the effect of proteases considerably longer time before loading the samples on the gel (compare Figs. 28 and 37).

Two isoforms of EF-Tu were found in membrane proteome of non-differentiating *Mycobacterium smegmatis*. Here we compared samples from exponential phase (22 hours of cultivation) with those from stationary phase (42 hours of cultivation) cultures grown in a liquid medium, since this strain is not capable of growing on glass beads. In both cultures

the amount and the ratio of EF-Tu isoforms did not differ, which probably reflected their simple life cycle compared to *Streptomyces*. However, we observed the presence of two isoforms of EF-Tu also in membranes of liquid grown non-differentiating culture of *S. coelicolor* (see Fig. 28). In Mycobacteria we observed in membrane fraction, similarly to Streptomycetes, characteristic EF-Tu isoform patterns, different from those in cell soluble fraction. There we found three isoforms. Since EF-Tu was already found to have some functions in the virulence of microorganisms like *Listeria monocytogens* (Archambaud *et al.* 2005), *Mycoplasma pneumonie* (Dallo *et al.* 2002) and *Lactobacillus johnsonii* (Granato *et al.* 2004) it is possible that also here in membranes of Mycobacteria it can have some additional function related to the virulence.

The charge heterogeneity of EF-Tu raises the question of the number and location of phosphorylations of the factor. Reversible protein phosphorylation is one of the major mechanisms of signal transduction in living organisms. We localized kinase(s) responsible for EF-Tu phosphorylation in both cell soluble and membrane fractions.

With the membrane and cell soluble fractions from the mycelium used as protein kinase sources, we could phosphorylate, in an *in vitro* assay using $\gamma[^{32}P]$ ATP as the phosphate donor, not only purified protein, but also host EF-Tu present in mentioned fractions. The assay is very simple and was optimised for the substrate/enzyme ratio as well as for other reaction components. However, like in the case of *E. coli* phosphorylation (Lippmann *et al.* 1993), the remaining problem is that there is always only a part of the factor phosphorylated. The reason for this limited phosphate transfer needs to be clarified. Using this assay, it should be possible to identify and purify the EF-Tu specific protein kinase and study it *in vitro*. Such an approach would help us to understand more about the role and mechanism of this posttranslational modification in the regulation of EF-Tu activity.

In the course of morphological differentiation of *S. coelicolor*, different isoform patterns of phosphorylated EF-Tu in the membrane fraction were found, whereas externally added EF-Tu contained always at least three isoforms because it is a mixture of factor from cytoplasm and membranes. EF-Tu was also phosphorylated in cytoplasm soluble fraction (S30), which means that kinase(s) able to phosphorylate EF-Tu is (are) present also in the cytoplasm of *S. coelicolor*. Since isoform pattern of phosphorylated EF-Tu of *S. coelicolor* was different in the membrane fraction (mostly two isoforms) from that in the cell soluble fraction (three isoforms), protein kinase(s) located in membranes might have different function than protein kinase(s) localized in the cytoplasm. Most probably

also the phosphorylation site of EF-Tu bound to membranes could be on a different residue than Thr_{385} in the conserved sequence region of EF-Tu. The identification of phosphorylated sites by mass spectrometry would be very helpful to clarify this problem.

The phosphorylation of the factor was detected also in dormant spores, where two isoforms of externally added factor and that of *S. coelicolor* were phosphorylated in an *in vitro* kinase reaction by crude cell extract. This shows for the first time in *Streptomyces* that their spores contain protein kinase able to phosphorylate its own EF-Tu as well as the externally added purified factor from *S. aureofaciens*. This indicates that phosphorylation of EF-Tu might play an important role in the germination of spores.

Contrary to the situation in *Streptomyces* we did not demonstrate phosphorylation of purified externally added *S. aureofaciens* EF-Tu in the membrane fraction of *M. smegmatis*. However, we found two phosphorylated isoforms of *M. smegmatis* EF-Tu in the membrane fraction, which could be explained by greater sequence differences between these two factors, or a different substrate specificity of protein kinases in membrane proteome of Mycobacteria. There were found several Thr residues in Mycobacterial EF-Tu sequence different from those in Streptomycetes factors (Table 7). This indirectly supports the hypothesis that this factor could play some role in the cell signalling of *Streptomyces*, because kinase phosphorylating EF-Tu in order to control its role in differentiation is not needed in non-differentiating Mycobacteria.

During development of *S. coelicolor*, there are apparent differences in membrane proteome and phospho proteome, which reflect changes connected with these processes. On the other hand, membrane proteome and phospho proteome of *M. smegmatis* is almost the same in young as well as in old culture indicating that there are not dramatic changes connected with aging of the culture.

Phosphorylation of EF-Tu depends also on the age of the culture. This was demonstrated in *S. granaticolor*, where we analysed whether the membrane fraction from the strain with inactivated PKG2 kinase grown in liquid culture is capable of phosphorylation of the factor. We also found that this first described *Streptomyces* trans membrane kinase is not responsible for EF-Tu phosphorylation. This might mean that there is (are) probably other kinase(s) doing this. Phosphorylation of EF-Tu might be also connected with biochemical differentiation since the factor is phosphorylated in a young culture before production of antibiotic granaticin and not in the old one producing granaticin in *S. granaticolor*.

The future goal in this project will be to identify and quantify all present posttranslational modifications of EF-Tu related to the cell development and differentiation. While phosphorylation on Thr_{385} residue is conserved and the region has probably permanent role in the regulation of EF-Tu activity, most probably in the control of protein synthesis, some other posttranslational modifications of EF-Tu might temporarily direct its function related to the cell differentiation. Primary sequence identification of EF-Tu isoforms should help us to reveal the overall physiological meaning of modifications of the factor in *Streptomyces* differentiation. Modification of EF-Tu could be for instance a way to change its specificity in order to increase its affinity to a rare tRNA species, such as leu-$tRNA_{TTA}$ (BldA) in *Streptomyces*, which seems to play an important role in the regulation of differentiation (see Chapter2), (Chater 1993). By this way, a special "translation compartment" could be created in which TTA codon directed regulation could take place, analogous to selenocystein incorporation mechanism (Baron and Bock 1991). Such modification of EF-Tu would make it in complex with leu-$tRNA_{TTA}$ distinguishable from all other ternary complexes (Ueda *et al.* 1992) and thus it could interact preferentially with UUA codons.

6. CONCLUSIONS

1) Although we observed few differences between EF-Tus from two strains of *Streptomyces aureofaciens*, its amino acid sequence was found to be identical. This means that data from *in vitro* biochemical experiments with tetracycline producing mutant strain *S. aureofaciens* 84/25 are valid also for the wild type *S. aureofaciens* ATCC 10762.
2) Obtaining the sequence of *tuf*1 gene coding for active EF-Tu in *Streptomyces aureofaciens* permitted us to create and compare 3D model structures of EF-Tu from *S. aureofaciens* with *S. coelicolor* and *M. smegmatis*. We have found that certain small part of EF-Tu from Streptomycetes surface structure differs, which might be a ground for differences in aggregation. The differences between the structure of Streptomycetes EF-Tu and that of *M. smegmatis* are much more pronounced.
3) EF-Tu associated with the cell membrane of *S. coelicolor* undergoes posttranslational modification(s) during differentiation, which might allow the protein to accept and transmit signals from the environment. We focused our attention on the phosphorylation and identified very variable pattern of EF-Tu phosphorylated isoforms, which was changing dramatically with the developmental stage. These isoforms might represent phosphorylations or other, yet unknown, posttranslational modifications of EF-Tu controlling its function in the cell differentiation, intracellular or extracellular signalling or other functions in the cell.
4) We showed that spores of *S. coelicolor* contain protein kinase able to phosphorylate its own EF-Tu as well as externally added purified factor from *S. aureofaciens*. This indicates that phosphorylation of EF-Tu might play an important role in the germination of spores.
5) We found that in Streptomycetes membrane fraction there is present kinase(s) capable of phosphorylation of both, its own, as well as an externally added EF-Tu from other strains, whereas *Mycobacterium* membranes contain protein kinase phosphorylating only its own EF-Tu.
6) We observed phosphorylation of EF-Tu also in *Streptomyces granaticolor*, where phosphorylation of this protein significantly depended on the age of liquid culture. Transmembrane kinase PKG2 is not involved in phosphorylation of this protein.

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Errata

Errata contain supplements, corrections and explanations of inaccuracies stated below.

Page 10. **1. Introduction**

The aims of the work

Part 1:

- Isolation of EF-Tu from two closely related strains of *Streptomyces aureofaciens* 84/25 and ATCC 10762, with the aim to compare conditions for spontaneous aggregation.
- To determine DNA sequence of *tuf1* gene coding for EF-Tu of *S. aureofaciens* ATCC 10762 and building of 3D molecular models of EF-Tu from *S. aureofaciens*, *Streptomyces coelicolor* and *Mycobacterium smegmatis* as a representative of non-differentiating Actinomycetes.

Part 2:

- Analysis of distribution and heterogeneity of EF-Tu in cell free factions of *S. coelicolor* and non-differentiating Actinomycete *M. smegmatis*.
- Characterisation of substrate specificity of transmembrane protein kinase PKG2 of *Streptomyces granaticolor*.
- Analysis of changes in membrane proteome of *S. coelicolor* during the differentiation and their comparison with non-differentiating Actinomycete *M. smegmatis*.
- Analysis of membrane phospho proteome changes in *S. coelicolor* during the differentiation and comparison with non-differentiating Actinomycete *M. smegmatis*.
- Comparison of an *in vitro* phosphorylation of externally added EF-Tu from *S. aureofaciens* and own *S. coelicolor* EF-Tu in membrane fraction.

Page 15. 2.1.7. Three-dimensional structures and crystallography

EF-Tu interacts with proteins, nucleic acids and nucleotides, making this molecule well suited as a model system for the study of these interactions (Kjeldgaard and Nyborg 1992). The N-terminal part of EF-Ts interacts with domain 1 of EF-Tu, while the C-terminal part interacts with domain 3. The C-terminal part has a protruding helix hairpin, which forms part of the interface to another pseudo-symmetric monomer. The resulting dimer of EF-Ts thus has two binding sites for EF-Tu on the same surface of the dimer (Nyborg and Liljas 1998). Stoichiometry of that complex then is EF-Tu:(EF-Ts)2:EF-Tu.

The crystal structure of intact elongation factor Tu from *Thermus thermophilus* (Berchtold *et al.* 1993), structure of complex between elongation factor Tu, Phe-tRNA and GTP (Bilgin *et al.* 1998) and that of the quaternary complex of Phe-tRNA:kirromycin:EF-Tu:GDPNP (Kristensen *et al.* 1996) from *E. coli* were resolved. They showed that EF-Tu and GTP form a 1:1 complex with aminoacyl-tRNA. The GTPase region of EF-Tu, interaction sites for the ribosome and that for tRNA are now well defined. The 3D structure of the ternary complex shows striking similarity to that of another prokaryotic elongation factor, EF-G (Clark and Nyborg 1997).

Dramatic conformational changes in EF-Tu occur when GTP is hydrolysed to GDP (Kjeldgaard *et al.* 1993). The most affected residues are called switch regions. A comparison of the switch I region in GTP and GDP conformation revealed that the segment of six amino acids completely converts part of switch I from an α helix in the GTP complex to β secondary structure in the GDP form (Abel *et al.* 1996). This α to β switch in EF-Tu may represent a prototypical activation mechanism for other protein families and is involved in triggering the release of tRNA and EF-Tu from the ribosome (Polekhina *et al.* 1996).

In *E. coli* EF-Tu domains 2 and 3 are absolutely required for establishment of the physiological affinity of EF-Tu for GDP and GTP, but they put some constraints on its GTPase activity. In contrast, the physiological affinity of EF-Tu from *B. stearothermophilus* for GDP/GTP appears to be the function of the G-domain itself, domains 2 and 3 do not change the nucleotide binding parameters, and they stimulate its GTPase activity (Sanderova and Jonak 2005).

Page 19. 2.1.8. Posttranslational modifications of EF-Tu and translation regulation

Because the Thr_{382} is located at a strategic position in the 1 and 3 domain interface (Fig. A), intercalation of the charged phosphate group changes the interaction between both domains, which abolishes its ability to bind aa-tRNA and keeps it in „GDP like conformation" (Alexander *et al.* 1995).

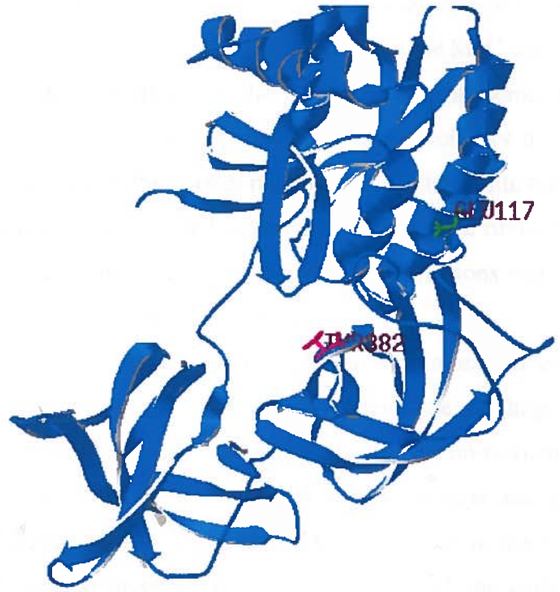


Figure A. Thr_{382} (red) from the third domain is bound by hydrogen bond to Glu_{117} (green).

Page 21. 2.1.11. Relative concentration and distribution of EF-Tu in bacterial cells

In *E. coli* (An *et al.* 1982), *B. subtilis*, *B. stearothermophilus* (Krasny *et al.* 2000) and *S. ramocissimus* (Tielman *et al.* 1997) there is in addition to the *str* operon's major promoter another, internal promoter. In *E. coli* this promoter, which can be used for the transcription of *tufA*, lies within *fus* gene, about 50 base pairs upstream from its 3' end and 120 base pairs from the start codon of *tufA* (An *et al.* 1982). The operon containing *tufB* has also at least one internal promoter, which is about one-tenth as active as the major promoter. In contrast to the situation in *E. coli*, where the ratio between the transcription of *tufA* gene to the whole *str* operon product is 7:1, in *B. stearothermophilus* it is about 10:1 in favour of the *tuf* gene transcript (Krasny *et al.* 2000). The activity of this *tuf* promoter is stimulated by the A/T-rich block.

Page 25. **2.1.12. Physiological functions of EF-Tu**

Many GTP-binding proteins have an absolute requirement for a divalent ion, usually Mg^{2+}, as a cofactor in the enzymatic reactions. Mg^{2+} ions are essential for either the binding of the nucleotide, GTP hydrolysis or the structural integrity of the protein. The rate constant of fast GTPase of EF-Tu on the ribosome also strongly depends on the Mg^{2+} concentration (Pape *et al.* 1998). However, the dissociation of EF-Tu·GDP from the ribosome is practically independent on the Mg^{2+} concentration.

Binding of tRNA to the A site of the ribosome is determined by a number of interactions that are responsible for the specificity of aminoacyl-tRNA selection and maintenance of the correct reading frame. The A-site binding is a multistep process that requires conformational adjustments of both the ribosome and tRNA. Purines stabilize codon anticodon interaction as well as interactions with the ribosome due to a stronger stacking (Konevega *et al.* 2004).

The GTPase activity of GTP-binding proteins is enhanced by proteins that make contact with the GTPase at the nucleotide binding pocket, thereby inducing the catalytically active conformation. In the codon-recognition complex ribosomal RNA contacts EF-Tu at the nucleotide-binding pocket and this contact may play a role in stabilizing the switch regions of the G domain in the catalytically active conformation and may be involved in the transmission of the codon-recognition signal and/or in activation of the GTPase of EF-Tu (Stark *et al.* 2002). The essential role of intact tRNA for GTPase activation may be due to a direct effect of tRNA on the arrangement of the effector loop, the tRNA may stabilize EF-Tu in the position required to establish activating interactions. Thus, ribosome makes contact with aa-tRNA in codon-recognition complex and may also be important for GTPase activation by precise positioning of EF-Tu for the reaction (Stark *et al.* 2002).

Binding of aminoglycoside antibiotics to 16S ribosomal RNA induces a particular structure of the decoding centre and increases the misincorporation of near-cognate amino acids. This is due to the stabilization of the near-cognate codon recognition complex (Pape *et al.* 2000). The efficiency of near-cognate aa-tRNA rejection in initial selection and proofreading is determined by both the lower stability of the codon–anticodon complex and slower forward reactions (GTPase activation and accommodation), compared to the cognate situation (Pape *et al.* 2000).

One of the most important aspects of the translocation mechanism is coupling of tRNA movement to mRNA movement. Failure of this process would result in a shift in

the translational reading frame, almost always causing premature termination at an out-of frame stop codon (Noller *et al.* 2002).

The ppGpp has been reported to increase the accuracy of translation *in vivo* (Rojas *et al.* 1984). Under *in vitro* conditions, it was shown that this molecule slows the rate of incorporation of amino acids into the protein through association with EF-Tu. EF-Tu.ppGpp complex can re-associate with the ribosome and prevent peptide bond formation. However, later it was found that ppGpp has a little or no direct effect on translation rate or fidelity. ppGpp inhibits mRNA synthesis, making mRNA limiting for translation during amino acid starvation. The reduced level of mRNA thereby reduces the severity of the aminoacyl-tRNA limitation and, in turn, mistranslation is avoided (Sorensen *et al.* 1994).

Page 27. **2.1.13. EF-Tu multifunctionality**

Initiation of ribosomal RNA synthesis is regulated also by the level of initiating nucleotide triphosphates (NTP). *E. coli* rRNA promoters require high initiating NTP concentrations for efficient transcription because they form unusually short-lived complexes with RNA polymerase. High concentrations of initiating NTP (ATP or GTP) are needed to bind to and stabilize the open initiation complex (Gaal *et al.* 1997). Six out of the seven *E. coli* rRNA promoters begin transcription with ATP, only one starts with GTP. In contrast to *E. coli,* the initiating NTP for transcription of *B. subtilis* rRNA is GTP (Krasny and Gourse 2004).

Page 28. **2.1.14. EF-Tu inhibitors**

Kirromycin is produced by *Streptomyces collinus* (Wolf *et al.* 1972) and interacts with EF-Tu (Wolf *et al.* 1974), possibly at the EF-Ts binding site, thereby inducing a conformational transition similar to that induced by EF-Ts. Kirromycin thus inhibits protein synthesis by preventing EF-Ts interaction with EF-Tu (Blumenthal *et al.* 1977).

Page 32. **2.2.1. The ecological niche of Streptomycetes**

In the absence of nutrients, needed for the effective regulation of pH adaptation, *Streptomyces* may not be able to grow especially at pH values below 7. The high organic load enables the functional intracellular pH regulation, and activates the growth of saprophytic Streptomycetes at low pH values (Kontro *et al.* 2005). However, pH has relatively small and variable effects on sporulation.

Page 35. **2.2.3. Morphological differentiation**

Bacterial death phenomena in communities appear to be active processes related to a multicellularity trait likewise subject to environmental factors and developmental processes. Recently it was shown that *Streptomyces* pass two death rounds during the development (Manteca *et al.* 2005). The first takes place very soon after the germination of spores and affects the young compartmentalized mycelium, whilst the second affects well developed substrate hyphae (Manteca *et al.* 2005). Most cells in substrate mycelium die during aerial mycelium formation, because the aerial mycelium reuses material first assimilated into the substrate mycelium. It is therefore conceivable that many hydrolytic enzymes, such as proteases, nucleases, and lipases, required for the degradation of cytoplasmic contents are produced at a specific time at the beginning of aerial mycelium formation (Tomono *et al.* 2005). Live and dead segments are present within the same hyphae in a very regular pattern. Subsequently, the remaining mycelium grows in successive waves, which vary according to the density of the spore inoculum. Aerial mycelium develops initially in the form of islands or circles. Further mycelial development occurs between the islands until the plate surface is totally covered (Manteca *et al.* 2005).

Page 39 and 43. **2.2.6. Regulation of *Streptomyces* differentiation**

Transcription of *AdpA*, a regulatory gene needed for colony morphogenesis in *Streptomyces coelicolor*, is activated during aerial hyphae formation and its TTA codon is required for aerial mycelium formation (Nguyen *et al.* 2003). AdpA is a positive regulator of the *melC* operon and so it is needed for melanogenesis. *mel* gene is not expressed in a *bldA* mutant and hence is likely to be controlled at the translational level via *bldA* tRNA (Zhu *et al.* 2005). *AdpA* is also a key transcriptional factor in the A-factor regulatory cascade in *S. griseus*. A-factor is essential for both streptomycin biosynthesis (Tomono *et al.* 2005) and aerial mycelium formation. AdpA activates a number of genes required for physiological and morphological differentiation and is self-controlled (Kato *et al.* 2005). Thus the intracellular concentration of A-factor determines the timing of secondary metabolism and morphological differentiation and contributes to coordination of gene expression in physically separate cells in a single hypha rather than between neighbouring hyphae. Therefore this factor resembles hormones in higher eukaryotes and is called a "microbial hormone" (Ohnishi *et al.* 2005). In contrast with A-factor, most γ-butyrolactons of other *Streptomyces* species are

involved only in a secondary metabolism and produced in a development-dependent manner, but not in a growth-dependent manner. AdpA is also a master regulator for the three extracellular chymotrypsin-type proteases, which probably play an important role during the differentiation (Tomono *et al.* 2005).

In *S. ansochromogenes sanG* gene encodes a transcriptional activator important for antibiotic nikkomycin biosynthesis that, unusually, also has pleiotropic effects on secondary metabolism and development, since *sanG* disruption abolishes nikkomycin biosynthesis, reduces sporulation, and leads to brown pigment accumulation (Liu *et al.* 2005). Transcription of this gene is growth phase-regulated. Some bald mutants of *S. ansochromogenes* have lost nikkomycin production, because two rare TTA codons are present in *sanG*.

Comprehensive cross-feeding tests among 76 *Streptomyces* strains showed that more than 20 % of the strains showed response(s) to a putative metabolite(s) excreted by another strain and exhibited precocious colony development and/or enhanced secondary metabolite formation. These stimulatory events between different species may include those involving the function of a specific metabolite. For instance the substance produced by *S. griseus* that stimulates the growth and development of *S. tanashiensis* is the siderophore desferrioxamine E (Yamanaka *et al.* 2005). In *Streptomyces* species, siderophores have a role in development as well as growth and their production is under complex regulation that links not only to ferric limitation but also to carbohydrate metabolism.

Gene expression in bacteria in response to various environmental and endogenous signals is coordinated by alternative sigma factors. *S. coelicolor* harbours some 66 sigma factors, which support its complex life cycle. σ^B is a master regulator for osmotic stress response, governing induction of more than 280 genes. Upon osmotic challenge it triggers the synthesis of at least two of its paralogues, σ^L and σ^M, which are also involved in the process of morphological differentiation. The phenotype of each sigma mutant suggested a sequential action in morphological differentiation: σ^B in forming aerial mycelium, σ^L in forming spores and σ^M for efficient sporulation. σ^B also contributes to protect cells against oxidative damage during ordinary growth as well as under osmotic stress (Lee *et al.* 2005).

The *rep* gene, derived from an environmental library, was shown both to accelerate sporulation and to enhance production levels of secondary metabolites, specifically

endogenous and heterologous antibiotics (Martinez *et al.* 2005). *Streptomyces* strains bearing this sequence can thus be used not only as improved production hosts for endogenous secondary metabolites but also for screening libraries of soil DNA samples for heterologous secondary metabolites, thereby enhancing our ability to detect novel compounds produced in environmental libraries.

Page 49. **2.3.5. EF-Tu in Mycobacteria**

M. avium overexpresses proteins within the macrophages that are involved in fatty acids metabolism (FadE2, FixA), cell wall synthesis (KasA), and protein synthesis (EF-Tu). These four proteins may be involved in the adaptation and survival of these bacteria within human macrophages. *M. avium* arrest of phagosome maturation is essential for growth inside the macrophage and requires accessibility to iron. It is known that *M. tuberculosis* EF-Tu is upregulated by high iron concentrations. Then overexpression of *M. avium* EF-Tu may be related to iron uptake by the Mycobacteria inside the phagosome. FadE2 and FixA can be sequentially involved in the β-oxidation of fatty acids, which are a major source of carbon and energy for Mycobacteria.

3. Materials and Methods

Page 51. **3.1. Materials**

Chemicals:

[^{3}H] GDP, (AMERSHAM)

[γ-^{32}P] ATP, (AMERSHAM)

β-mercaptoethanol, (KOCH LIGHT LAB.)

7H9 and 7H10 medium, (CHEMOS)

Acetic acid, (SERVA)

Acrylamide 2X, (SERVA)

$AgNO_3$, (CHEMAPOL)

Ampholine, (SIGMA)

APS, (SERVA)

ATP, (SIGMA)

BamH1, (NEB)

BCA protein assay kit, (PIERCE)

Bis-acrylamide 2X, (SERVA)

Cassamino acid, (DIFCO LABORATORIES)

CHAPS, (SERVA)

KCl, (SERVA)

Chlorophorm, (SERVA)

Coomassie Blue R-250, (MERCK)

DEAE-Sepharose Cl-6B, (PHARMACIA-LKB)

Dialysis tubing, (SERVA)

Dithiothreitol, (SIGMA)

dNTP, (PROMEGA)

Ethanol, (MERCK)

Formaldehyde, (CHEMAPOL)

GDP, (SERVA)

Glucose, (LACHEMA)

Glutaraldehyde, (SIGMA)

Glycerol, (LACHEMA)

Glycine, (SERVA)

NaOH, (SERVA)

Iodoacetamide, (SIGMA)

K_2CO_3, (SIGMA)

EDTA, (SERVA)

LKB 4/6 Ampholite, (LKB)

LKB 5/7 Ampholite, (LKB)

Lysozyme, (SIGMA-ALDRICH)

$MgCl_2$, (TAKARA)

Na_2CO_3, (SERVA)

$Na_2S_2O_3$, (FLUKA)

NaF, (SIGMA)

Natrium acetate, (FLUKA)

NH_4Cl, (LACHEMA)

Nitrocellulose membrane, (LACOMED)

Oligonucleotides, (VBC)

PCR buffer, (PROMEGA)

Pharmalite 3-10, (SIGMA)

Phenol, (SIGMA)

Plasmid purification kit, (QIAGEN)

PMSF, (SERVA)

Potassium tetrathionate, (FLUKA)

Primary antibody, (MICROBIOLOGY INSTITUTE)

Protease inhibitor cocktail, (SIGMA)

Protease inhibitors set, (ROCHE)

Proteinase K, (SIGMA-ALDRICH)

RNase, (PROMEGA)

SDS, (SERVA)

Secondary antibody, (AMERSHAM)

Sephadex G-200, (PHARMACIA)

Sigma 3/10 Ampholite, (SIGMA)

Sigma 5/7 Ampholite, (SIGMA)

Skim milk powder, (OXOID)

Super signal West Pico, (PIERCE)

Taq polymerase, (PROMEGA)

TEMED, (BIORAD)

Tris HCl, (SERVA)

Triton X-100, (SIGMA)

Tween 20, (SERVA)

Tween 50, (SERVA)

Urea, (SERVA)

Yeast extract, (OXOID)

Other chemicals were common accessible substances of analytical purity.

Page 51. **3.1.1. Bacterial strains and plasmids**

Mycobacterium smegmatis (mc^2 155), *Streptomyces granaticolor* (ETH 7437), *Streptomyces coelicolor* A3(2) J1501 (John Innes Center, Norwich, United Kingdom), *Streptomyces collinus* (ETH 24318) and *Streptomyces aureofaciens* ATCC 10762 and 84/25, a tetracycline high-producing mutant, were from the collection of microorganisms of the Institute of Microbiology (MBU), Prague. All of these strains were stored in 20% glycerol at –80 °C. Plasmid bluescript pBS-SK was from the Institute of Molecular biology, Bratislava and was stored in buffer TE.

Page 53. **3.1.3. Buffers and solutions**

Sample buffer for 1D electrophoresis: 0.012M Tris HCl (pH 6.8), 0.05% glycerol, 0.4% SDS, 0.01% (v/v) β–mercaptoethanol, 0.02% bromphenol blue

Lysis buffer for 2D electrophoresis: 3% urea, 0.25% CHAPS, 0.02% (v/v) pharmalyte 3-10, 0.06% DTT, few grains of bromphenol blue

TAE buffer: 0.48% Tris base, 0.11% (v/v) acetic acid, 0.07% $Na_2EDTA.2H_2O$

Binding buffer for GDP binding assay: 0.01M Tris HCl, 0.2% magnesium acetate.$4H_2O$, 0.07% (v/v) β–mercaptoethanol, pH 7.5

Page 54. **3.2.1. Cultivation of *Streptomyces* mycelium in liquid culture**

After 2 days of aerated cultivation at 28 °C, 3 – 5 ml of mycelium was used as inoculum into 60 ml of fresh medium, where it grew for 20 hours.

Page 56. **3.2.4. Purification of *Streptomyces aureofaciens* elongation factor Tu**

The protein spontaneously aggregates when its concentration in solution is increased. In experiments it was then solubilized by dilution, increased temperature or presence of EF-Ts.

Page 62. **3.2.9. Two-Dimensional Polyacrylamide Gel Electrophoresis**

Running conditions:

Isoelectric focussing was set to 18,000 V/hr but the real values started from 0 V/hr and increased until they reached set values during the last 30 min of the run. At this setting the length of the run was 17 hours 30 minutes.

Page 65. **3.2.10. Silver Staining**

Reagents:

Fix solution 1: 40% (v/v) ethanol, 10% (v/v) acetic acid

Fix solution 2: 0.5% glutaraldehyde, 30% (v/v) ethanol, 0.25% potassium tetrathionate, 6.8% sodium acetate

Silver nitrate solution: 0.2% silver nitrate, 0.025% (v/v) formaldehyde

Developer solution: 3% potassium carbonate, 0.00075% sodium thiosulphate, 0.015% formaldehyde

Page 69. **3.2.13. *In vitro* DNA manipulations**

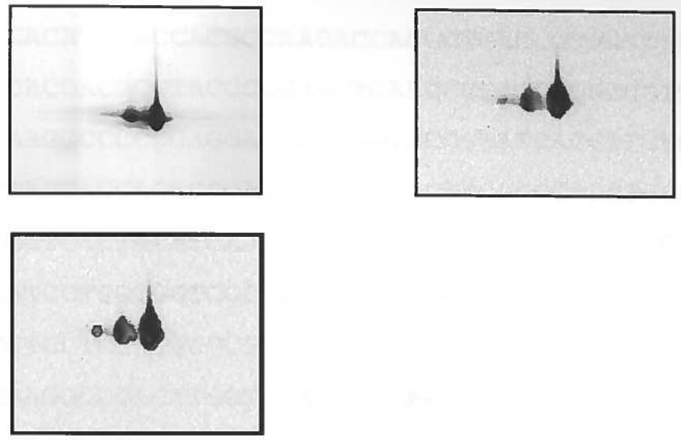
Chlorophorm was removed by evaporation in exhauster (5 min).

4. Results

Page 70. **4.1. Functional and structural aspects of elongation factor Tu**

Page 73; Figure 9. SDS-PAGE of 2 μg of purified EF-Tu from *S. aureofaciens* 84/25 (band on the left) and *S. aureofaciens* ATCC 10762 (band on the right).

Page 74; Figure 11. We do not have the direct evidence that it is a multimer of EF-Tu. However, this spot was detected with polyclonal antibody against EF-Tu in a number of cases. The molecular weight determined from protein mobility in SDS gel is more than 200 kDa.



Page 75; Figure 12. At the top is EF-Tu from *S. aureofaciens* ATCC 10762, silver stained gel (on the left) and detection of EF-Tu by antibody against EF-Tu (on the right), pI 4 – 7. At the bottom there is densitometry pattern of purified EF-Tu isoforms. The first spot represents 4 %, second 27.4 % and third 68.6 % of all the spots.

Page 75; Figure 13. This spontaneous degradation of EF-Tu from *S. aureofaciens* ATCC 10762 is probably caused by protease contamination in the preparation.

Page 77; Figure 14. This apparent instability of EF-Tu from *S. aureofaciens* ATCC 10762 is probably also caused by protease contamination in the preparation or by greater volume of the protein.

Page 76; Table 2. This Table is just to show the sequence of EF-Tu fragments observed on SDS gel. For this reason there are not included the smallest fragments, which would not be detected on SDS gel.

GTGGCCAAGGCGAAGTTCGAGCGGACGAAGCCCCACGTCAACATCGGCACC

ATCGGTCACATCGACCACGGCAAGACCACGCTGACCGCGGCCATCACCAAG

GTGCTGCACGACGCGTACCCGGAGATCAACCCCTTCACGCCGTTCGACCAG

ATCGACAAGGCCCCGGAGGAGCGTCAGCGCGGTATCACCATCTCGATCGCG

CACGTCGAGTACCAGACCGAGGCGCGTCACTACGCCCACGTCGACTGCCCG

GGTCACGCTGACTACATCAAGAACATGATCACCGGTGCCGCCCAGATGGAC

GGCGCCATCCTGGTGGTCGCCGCCACCGACGGCCCGATGCCGCAGACCAAG

GAGCACGTCCTCCTGGCCCGCCAGGTCGGCGTTCCGTACATCGTCGTCGCC

CTGAACAAGGCCGACATGGTGGACGACGAGGAGATCCTGGAGCTCGTCGAG

CTCGAGGTCCGCGAGCTCCTCTCGGAGTACGAGTTCCCGGGCGACGACCTG

CCGGTCGTCCGCGTCTCCGCCCTGAAGGCCCTCGAGGGCGACAAGGAGTGG

GGCGAGAAGCTCCTCGGCCTCATGCACGCGGTCGACGAGAACATCCCCACC

CCGGCCCGCGCCGTGGACCAGCCGTTCCTGATGCCGATCGAGGACGTCTTC

ACGATCACCGGTCGTGGCACCGTCGTCACCGGTCGTATCGAGCGCGGCATC

CTCAAGGTCAACGAGACCGTCGACATCATCGGCATCAAGACCGAGAAGACC

ACCACCACGGTCACCGGCATCGAGATGTTCCGCAAGCTGCTCGACGAGGGC

CAGGCCGGTGAGAACGTCGGTCTGCTGCTCCGTGGCATCAAGCGCGAGGAC

GTCGAGCGCGGCCAGGTCATCATCAAGCCGGGTTCGGTTACCCCGCACACC

GACTTCGAGGCCCAGGCCTACATCCTGTCGAAGGACGAGGGTGGCCGCCAC

ACCCCGTTCTTCAACAACTACCGCCCGCAGTTCTACTTCCGTACCACGGAC

GTGACCGGCGTCGTGACCCTCCCCAAAGGCACCGAGATGGTCATGCCGGGC

GACAACACCGCCATGACCGTCGCGCTGATCCAGCCCGTCGCCATGGAGGAG

GGCCTGAAGTTCGCCATCCGTGAGGGTGGCCGTACCGTCGGCGCCGGCCAG

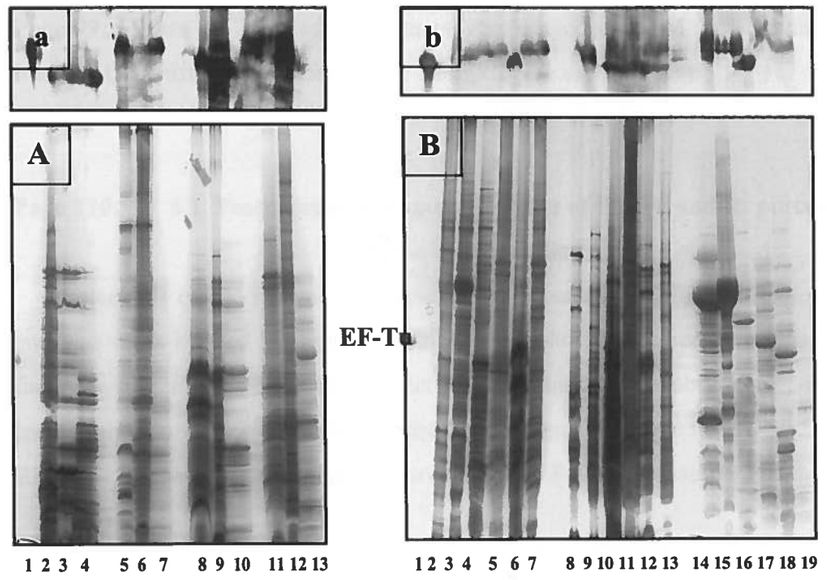
GTCACCAAGATCGTCAAGTAATTCAACG

Page 81; Table 3. Nucleotide sequence of *tuf1* gene from *S. aureofaciens* ATCC 10762. Nucleotides, which differ from *tuf1* gene of *S. aureofaciens* 84/25 are in blue. Start and stop codons are in red.

Page 84. 4.2. Possible role of EF-Tu in cell signalling and regulation of the cell differentiation

Page 87. 4.2.1. Preparation of tools and building of experimental strategy

Mycobacterium smegmatis is non-differentiating Actinomycete, which does not sporulate. Here were analysed and compared posttranslational modifications of EF-Tu in cells from exponential (22 hours of cultivation) and stationary (42 hours of cultivation) phase of growth. The shapes of bacteria emerge in the scanning electron microscope identical and in the stationary phase, there is much more cells present and these appear in clumps (Fig. 24). This is due to a slow outgrowth of *M. smegmatis* culture.



Page 88; Figure 25. A, a: Membrane, ribosomal and S150 fractions numbered respectively, from the following sources: *E. coli* (2 – 4), *Mycobacterium smegmatis* (5—7), *S. aureofaciens* 84/25 (8—10) and *S. coelicolor* (11—13). B, b: Samples from *S. aureofaciens* 3239, *S. granaticolor*, *S. collinus*, *S. coelicolor*, *S. aureofaciens* 84/25, *S. lividans* respectively representing membrane (2—7), ribosomal (8—13) and S150 (14—19) fraction. (1) – standard of EF-Tu. Silver stained gels (A, B). EF-Tu in all tested fractions was detected by Western blot technique (a, b).

Fig. 25 should demonstrate that our polyclonal antibody against EF-Tu from *S. aureofaciens* detects EF-Tu also in other bacterial species. It also shows that EF-Tu is present not only in cytoplasm and on ribosomes as would be expected, but also in membrane fraction.

Page 91. 4.2.3. Phosphorylation of EF-Tu during growth and differentiation of *Actinomyces*

All further analysed phospho proteomes were obtained by *in vitro* kinase reactions, where the substrate was EF-Tu, potential donors of protein kinases were membrane or cystoplamic fractions and donor of phosphate group was [γ- ^{32}P] ATP. Phosphorylated proteins were detected in phosphor imager (see 3.2.6.for details).

Page 99; Figure 34. Purified membranes were treated with 1M ammonium chloride in fractionation buffer F. Membranes are from *S. coelicolor*.

5. Discussion

Page 110. 5.2. Posttranslational modifications of EF-Tu and its potential role in the cell signalling

Proteolysis of EF-Tu was also observed in other stages of *S. coelicolor* life cycle and was most marked in samples from *in vitro* phosphorylation reactions, where the factor is subject of the possible effect of proteases considerably longer time before loading the samples on the gel (compare Figs. 28 and 37, where in Fig. 37 *S. coelicolor* EF-Tu is at lower position than *S. aureofaciens* EF-Tu, because part of the protein is digested).

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