

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH. D.)

**Study of the sporulation, pathogenicity and taxonomic
identification of the factor C producer *Streptomyces griseus* 45H
strain**

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ABBREVIATIONS

<i>aacC4</i>	aminoglycoside-3-acetyltransferase IV gene
AdpA	A-factor dependent protein
Apr	apramycin
<i>facC</i>	factor C gene
MM	minimal medium
<i>necI</i>	necrogenic factor gene
PAI	large pathogenicity island
PCR	polymerase chain reaction
PNB	p-nitrophenyl butyrate
rRNA	ribosomal RNA
Thio ^S	thiostreptone sensitive
Apr ^R	apramycin resistant
45H	<i>S. griseus</i> (<i>S. albidoflavus</i>) 45H strain
45H-B	null mutant of <i>S. griseus</i> (<i>S. albidoflavus</i>) 45H strain
52-1	<i>S. griseus</i> 52-1 strain
CFBP4501	<i>S. europaeiscabiei</i> CFBP4501 scab causing strain

1. INTRODUCTION

Members of the Gram-positive multicellular bacterial genus *Streptomyces* are of both academic and applied interest: they show complex morphological differentiation culminating in sporulation and produce a variety of secondary metabolites including over 60% of all known antibiotics and a wide range of industrial enzymes. The complex life cycle is often controlled by extracellular autoregulatory molecules. A-factor (2-isocapryloyl-(3R)-hydroxymethyl- γ -butyrolactone), the best known autoregulator, regulates aerial mycelium formation and secondary metabolite biosynthesis in *Streptomyces griseus*.

Another interesting autoregulator is the secreted signaling protein factor C, isolated from the culture fluid of a strain originally identified as *Streptomyces griseus* 45H. The factor C producer strain was isolated during a mutagenesis program aimed at the isolation of morphological and streptomycin non-producer mutants of the industrial streptomycin producer *S. griseus* 52-1. The assumed origin of strain 45H has often been questioned because the two strains differ in several substantial properties and based on the facts that *facC* gene was not detected in the genomic DNA of strain 52-1 by Southern hybridization. However the restriction digestion and hybridization patterns of strain 45H and a laboratory strain of the flavofungin producer *Streptomyces flavofungini*, studied in the same time, are highly similar.

S. griseus 45H has been described as a strain that sporulates well both on solid medium and in liquid culture, the latter being a rather rare property of *Streptomyces* strains. The secreted factor C induces cytodifferentiation of *S. griseus* 52-1 and stimulates its sporulation in liquid culture. The expression of *facC* gene has also been studied in different *Streptomyces* strains, however the role of factor C in morphological differentiation of the producer 45H strain is not known in detail.

Southern hybridization and database searches revealed only very few *Streptomyces* strains with *facC* homologous genes. Interestingly, one of them is the best known plant pathogenic *S. scabies* which causes common scab disease on potato. However, potato scab is also caused by a small number of phylogenetically diverse *Streptomyces* species as some scab causing isolates have been identified as *S. albidoflavus*.

In potato scab pathogens, pathogenicity depends on PAI that carries thaxtomin biosynthetic genes, the *nec1* gene for a necrogenic factor and other putative virulence genes. In some strains (though apparently not in *S. scabies* itself) this PAI is transmissible into normally non-pathogenic streptomycetes, and can confer pathogenicity on the new host. Potato tuber periderm, with suberin in the cell walls, plays a very important role in protection against desiccation and pathogen invasion therefore production and secretion of cell wall-degrading enzymes like extracellular esterase are also important for plant pathogenic microorganisms.

Factor C was shown to be involved in the regulation of expression of several extracellular enzymes in *S. griseus* by inducing A-factor production and expression of the genes of the A-factor regulon. These extracellular enzymes use zinc ion as cofactor. Upstream of their genes there is at least one consensus binding site for AdpA, a key pleiotropic regulatory protein in different streptomycetes. The *S. scabies* extracellular esterase is also zinc activated, and upstream of its genetic determinant there is a protein binding site that is highly similar to the AdpA consensus binding site. Furthermore, *S. scabies* has a gene highly homologous to *afsA* encoding the key enzyme for A-factor synthesis. Thus, the esterase may be part of the large regulon of different genes subject to activation by AdpA.

Based on the above mentioned information we aimed the followings:

1. To study the relatedness of *S. griseus* 45H, *S. griseus* 52-1 and our laboratory strain *S. flavofungini* by 16S rRNA gene sequence comparison and to confirm the revealed taxonomic identification of strain 45H by Southern blotting.
2. To construct a *facC* null mutant of *S. griseus* 45H for study whether the morphological differentiation of producer strain 45H is blocked due to the lack of functional *facC* gene and which steps of differentiation are controlled by factor C.
3. Although the identity and mode of action of factor C has been the subject of many publications, the exact role of factor C as well as the regulatory cascade in which the factor C is involved has not been known yet in detail, therefore we made effort to find *facC* homologues in different microorganism.
4. To study whether the *necI* gene and supposedly the PAI is present in *S. griseus* 45H as well as the extracellular esterase corresponding to pathogenicity is produced by that strain and factor C might be involved in the regulation of expression of extracellular esterase and therefore of pathogenicity.
5. To test the pathogenicity of *S. griseus* 45H on potato microtubers compared with other pathogenic and non-pathogenic strains.

2. MATERIALS AND METHODS

2.1. Revising of taxonomic identification of *S. griseus* 45H

The relatedness of *S. griseus* 45H, the laboratory strain of *S. flavofungini* and *S. griseus* 52-1 strains was investigated by comparison of 16S rRNA gene sequences and the taxonomic identification of strain 45H was confirmed by Southern blotting. The PCR amplified 16S rRNA gene sequence was determined by direct sequencing in each strain and the 16S rRNA gene phylogenetic tree was generated by using Jukes and Cantor distances and the neighbour joining method. For Southern blotting the digoxigenin labelled (Dig Nucleid Acid Detection Kit, Boehringer Mannheim) 860 bp *EcoRV-SalI facC* fragment was used as a probe.

2.2. Construction and study of *facC* null mutant of *S. griseus* 45H

To construct a *facC* null mutant, *in vitro* insertional inactivation of *facC* gene was designed by placing an *aacC4* gene encoding Apr resistance into the cloned copy of *facC* carried on pSGF4 plasmid. The *Streptomyces* replication origin also carried on pSGF4 plasmid was eliminated to force homologous recombination between the chromosomal copy of *facC* and its inactivated form following the transformation. The resulted pSB51 plasmid carrying a *thio* selectable marker alongside the *facC* gene disrupted by *aacC4* gene was introduced into the *S. griseus* 45H protoplast by polyethylene glycol induced transformation. Thio^S, Apr^R colonies shown *bald* phenotype on the mutant selection plates were picked up and their genetic composition was analyzed by Southern blotting with the radioactive labelled 860 bp *EcoRV-SalI facC* fragment, extending from 835 bp upstream of the insertion position of the Apr resistance gene to 25 bp downstream of it was used as a probe.

2.3. Search for *facC* homologues

To find *facC* homologues, GenBank database and PHI-BLAST software were used.

2.4. Study of presence of *nec1* gene in *S. griseus* 45H and other *Streptomyces* strains

The presence of *nec1* gene in different *Streptomyces* strain - 45H, 45H-B, 52-1, CFBP4501 - was investigated by PCR reaction and Southern blotting. For amplification of *nec1* gene, we used the same primer sets and reaction were described previously to detect *nec1* gene in *S. scabies*. For Southern blotting, the PCR amplified CFBP4501 *nec1* gene was labelled by digoxigenin, using the Roche DIG DNA labeling and detection kit.

2.5. Study of extracellular esterase activity in *S. griseus* 45H and other *Streptomyces* strains

Streptomyces strains - 45H, 45H-B, 52-1, CFBP4501 - were grown in MM with or without zinc, simultaneously. Extracellular esterase activity was measured by a spectrophotometric assay using PNB as the substrate. The reaction was started by adding PNB to the supernatant of cell culture and hydrolysis was continuously monitored in a spectrophotometer at 420 nm for 10 min at room temperature. In a parallel experiment the supernatants were incubated at 60 °C for 10 min before the addition of PNB in order to test the presence of heat-stable esterase. The assay was repeated three times. Extracellular esterase activity was calculated for 1 mg dry weight of mycelium and expressed as $\text{nmolmin}^{-1}\text{mg}^{-1}$. The standard deviations were calculated and the significance of differences between data sets was tested using a t-test.

2.6. Potato microtuber pathogenicity test

To test bacterial pathogenicity, *in vitro* tubers of *Solanum tuberosum* L. cultivars (cv.) Rebeka, Desiree and Cleopatra as well as 45H, 45H-B, 52-1 and CFBP4501 cell suspension were used. Twelve microtubers of each cultivars were immersed in 20-20 ml bacterial suspension for 10 min. Five tubers were immersed in sterile distilled water as negative control. Tubers were placed in covered Petri dishes containing moist paper towels and incubated at 28 °C. The pathogenicity reaction was scored daily for 10 days. A symptom scale was adopted ranging from 0 to 4 (0 = no symptoms; 1 = white spots and big colonies around the lenticels; 2 = brown spots and scabs around the lenticels; 3 = scabs everywhere on the tuber; 4 = scabs, lesions and rotted tubers).

2.7. Other methods

For growing and handling of *Streptomyces* cultures, transformation of *Streptomyces* protoplasts, isolation of chromosomal DNA from *Streptomyces*, transformation of *E. coli* cells, isolation of plasmid DNA from *E. coli*, agarose gel electrophoresis, isolation of DNA from agarose gel, ligation of DNA fragments standard methods were used as described in the following manuals: Genetic Manipulation of *Streptomyces*, Practical *Streptomyces* Genetics and Molecular cloning, A Laboratory Manual, 3rd ed.

3. NEW RESULTS AND DISCUSSION

3.1. Revising of taxonomic identification of *S. griseus* 45H

The 16S rRNA gene sequences of *S. griseus* 45H, the laboratory strain of *S. flavofungini* and *S. griseus* 52-1 were determined by direct sequencing and a 16S rRNA gene phylogenetic tree of these strains, related streptomycetes and representative strains of the genus *Streptomyces* was constructed. The gene sequence for *S. griseus* 52-1 matches the database entries for *S. griseus* rRNA operons *rrnA-E* confirming that it belongs to the *S. griseus* group. The gene sequences for *S. griseus* 45H and our laboratory strain of *S. flavofungini* were identical, however differed from the sequences for *S. griseus* 52-1 and from deposited type strains of *S. flavofungini* NRRL B-12307 and NBRC 13371. On the other hand the sequences for *S. griseus* 45H and our laboratory strain of *S. flavofungini* were identical to the 16S rRNA gene sequences for members of the *S. albidoflavus* species group.

Since the strain 45H was identified as a member of the *S. albidoflavus* species, four members of that species were tested for *facC* gene by Southern blotting. Three strains gave strong signals, however the type strain, which is relatively distinct from the rest of the members of the species, did not give a signal. Previous studies indicated that *facC* is absent from many *Streptomyces* genomes, therefore it may be specifically associated with *S. albidoflavus* and related species group.

Our recent molecular data in line with previous findings indicate that strain 45H was probably picked up as a laboratory contaminant of the strain of *S. flavofungini*. Based on 16S rRNA gene sequences and Southern blotting, *S. griseus* 45H and *S. flavofungini* from our culture collection are clearly identified as the same and belong to the *S. albidoflavus* group. Therefore *S. griseus* 45H should be named as *Streptomyces albidoflavus* 45H in the future.

3.2. Construction and study of null mutant of *S. griseus* 45H

To study the role of factor C in cellular differentiation we made a null mutant of *S. griseus* 45H. Null mutation was designed by replacing the *facC* gene in the chromosome by its *in vitro* inactivated form via double crossing over. Thio^S, Apr^R colonies, the candidate gene replacement mutants, showed *bald* phenotype in agreement with our expectation. Several independent isolates were analyzed by Southern blot. The fact that we did not detect any signal in these independent isolates suggested that instead of the expected homologous recombination part of the target gene expected to show hybridisation (i.e. that to the left of the inserted resistance gene) was completely deleted. The non-sporulating *bald* phenotype of the mutant was restored to the wild-type phenotype when *facC* was reintroduced on a plasmid showing that the deletion of *facC* gene was responsible for the bald phenotype.

3.3. Search for *facC* homologues

Database searches revealed only very few *Streptomyces* strain, e.g. *Streptomyces albus*, *S. scabies* 87.22, *S. ambofaciens* with related, but somewhat diverged genes. Strains *S. coelicolor* A(3)2, *S. avermitilis* MA-4680 and *S. griseus* IFO 13350 does not contain *facC*-like gene. The *facC*-like genes in *S. scabies* and *S. ambofaciens* are located near to the terminus of the linear chromosomes, a region that is frequently involved in deletion, rearrangements and the acquisition of DNA by horizontal gene transfer. This could account for the sporadic occurrence of *facC* genes in diverse *Streptomyces* strains.

Other sequences related to *facC* gene can be found in three main phylogenetically distinct groups of microorganisms: frequently in low-GC Gram-positive bacteria, especially staphylococci, lactococci and their phages, where they are generally part of larger proteins, and occasionally in related actinomycetes, and – quite surprisingly – in some mycelial fungi.

3.4. Study of presence of *nec1* gene in *S. griseus* 45H and other *Streptomyces* strains

The *nec1* gene is highly conserved and usually located on PAI along with other virulence genes (e.g. the thaxtomin family). Using PCR, the *nec1* gene (and supposedly the PAI) was detected in the pathogen CFBP4501 but not in the other three studied strains. To test whether this negative result was due to sequence divergence of *nec1*, we used the PCR amplified fragment from CFB4501 as a probe in Southern hybridization. The *nec1* gene was detected by Southern hybridization in both 45H and its *bald* mutant but not in 52-1. Thus we think that *S. griseus* 45H carries at least some DNA corresponding to the PAI of well-characterised scab-inducing pathogens and the segment corresponding to *nec1* was not affected by the deletion of *facC* gene in 45H-B.

3.5. Study of extracellular esterase activity in *S. griseus* 45H and other *Streptomyces* strains

Extracellular esterase activity was studied in cultures grown on MM medium with or without zinc supplementation. The esterase activity was monitored in parallel in the supernatants with or without heat inactivation of the enzyme. The enzyme activity was relatively high and moderately high in strains CFBP4501 and 45H respectively. In these two strains the enzyme was inducible by zinc. In the *facC* null mutant 45H-B the activity was relatively low and was not inducible by zinc. 52-1 showed a low-level, not inducible esterase activity. The observed differences in all cases of the pair of samples compared were statistically significant at least at a level of $p=0.05$ or less. These results support the assumption that extracellular esterase is somehow regulated by the extracellular regulatory protein factor C.

3.6. Potato microtuber pathogenicity test

Four *Streptomyces* strains were investigated: the pathogen CFBP4501, the factor C producer 45H, its *facC* null mutant 45H-B and 52-1 as a supposedly negative control. Pathogenicity was estimated by a scoring system ranging from 0 to 4. Score below 1 was considered non-pathogenic, between 1 and 2 moderately pathogenic, and above 2 virulent. The CFBP4501 strain showed high virulence on all of three cultivars, while strain 52-1 as expected was non-pathogenic. Somewhat surprisingly but consistent with the report that some scab-inducing strains are *S. albidoflavus*, strain 45H showed moderate virulence on all of three cultivars. In line with esterase activity, *facC* null mutant showed moderate but reduced pathogenicity compared to strain 45H.

4. SUMMARY

Our recent molecular data in line with previous findings indicates that *S. griseus* 45H was probably picked up as a laboratory contaminant of the strain of *S. flavofungini*. Based on 16S rRNA gene sequences and Southern blotting, *S. griseus* 45H and *S. flavofungini* from our culture collection are clearly identified as the same and belong to the *S. albidoflavus* group. Therefore *S. griseus* 45H should be named as *Streptomyces albidoflavus* 45H in the future.

A *facC* null mutant of strain 45H were designed by replacing the *facC* gene in the chromosome by its *in vitro* inactivated form via homologous recombination. The candidate gene replacement mutants showed the expected *bald* phenotype; however, instead of the expected homologous recombination probably induced genome rearrangement happened that resulted in deletion to one side of the *facC* gene region as it was revealed by Southern hybridization. Deletions often occur in the terminal region of the unstable linear chromosomes hence a similar chromosomal localization would explain the loss of *facC* DNA in *S. albidoflavus* 45H. The resulting *bald* phenotype, and its complementation by the cloned *facC* gene, confirmed that factor C plays a key role in controlling cellular differentiation.

NecI was used as an indicator of the presence of the large PAI that confers pathogenicity to different *Streptomyces* strains. We showed by PCR amplification and Southern hybridization that the gene was present in *S. europaeiscabiei* CFBP4501, *S. griseus* 45H and 45H-B but absent in *S. griseus* 52-1. Probably, the null mutant was deleted not only for *facC*, but also for additional DNA, though some part of the PAI-related DNA (*necI*) was retained by the mutant.

We also found a good correlation between the production of a zinc-induced heat-stable extracellular esterase and the pathogenicity of the strains. Both the pathogenicity and esterase activity was decreased but not lost in null mutant and

which suggest that factor C has a possible regulatory role in the production of extracellular esterase and the change in esterase activity can not be attributed to codeletion of factor C gene and the esterase gene.

Combining the information regarding to the possible interaction between factor C and A-factor regulon as well as characteristics of extracellular enzymes regulated by factor C and the fact that upstream of extracellular esterase genetic determinant there is a protein binding site that is highly similar to the AdpA consensus binding site, we assume that factor C plays a regulatory role in the production of extracellular hydrolases and consequently cellular differentiation.

5. PUBLICATIONS

5.1. Publications subject to the thesis

- Kiss Zs**, Ward AC, Birkó Zs, Chater KF, Biró S (2008) *Streptomyces griseus* 45H, the producer of the extracellular autoregulator protein factor C, is a member of the species *Streptomyces albidoflavus*. *Int J Syst Evol Microbiol* 58: 1029-1031 (IF: 2.222)
- Kiss Zs**, Dobránszki J, Hudák I, Birkó Zs, Vargha Gy, Biró S (in press): The possible role of factor C in common scab disease development. *Acta Biol. Hung.* (IF: 0.619)

5.2. Other publications

- Kiss Zs**, Biró S (2002) A C faktor gén inaktiválása *Streptomyces griseus*ban kopasz fenotípust eredményez. A Magyar Biokémiai Egyesület Molekuláris Biológiai szakosztály 7. munkaértekezlete, Keszthely (poster)
- Biró S, Birkó Zs, **Kiss Zs**, Keller U (2002) Factor C, a secreted pleiotropic autoregulator of cell differentiation in *Streptomyces griseus*. 9th International Symposium of Industrial Microorganisms. Gyeongju, Korea (poster)
- Birkó Zs, **Kiss Zs**, Biró S (2003) Factor C, a secreted pleiotropic autoregulator of cell differentiation in *Streptomyces griseus*. *Streptomyces dissemination meeting*. University of Surrey, Guildford
- Birkó Zs, **Kiss Zs**, Biró S (2005) *Streptomyces*ek sejtdifferenciálódásának vizsgálata genomikai és proteomikai módszerekkel. VI. Magyar Genetikai Kongresszus, Eger (lecture)

- Birkó Zs, **Kiss Zs**, Biró S (2005) Study of the extracellular proteome of *Streptomyces griseus* during development. 1st Central European Forum for Microbiology (CEFORM) and the Annual Meeting of the Hungarian Society for Microbiology, Keszthely (poster)
- Kiss Zs**, Dobránszki J, Hudák I, Biró (2007) A C faktort termelő *Streptomyces griseus* 45H törzs patogenitásának vizsgálata burgonyán. VII. Magyar Genetikai Kongresszus – XIV. Sejt- és Fejlődésbiológiai Napok, Balatonfüred (poster)