Transcriptional and functional analysis of the gene for factor C, an extracellular signal protein involved in cytodifferentiation of Streptomyces griseus

Biro, S.; Birko, Z.; Wezel, G.P. van

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Transcriptional and functional analysis of the gene for factor C, an extracellular signal protein involved in cytodifferentiation of *Streptomyces griseus*

Sándor Biró[1,2,*], Zsuzsanna Birkó[1] & Gilles P. van Wezel[3]
[1]*Department of Human Genetics, Faculty of Medicine, Medical and Health Science Center, University of Debrecen, H-4012 Debrecen, Nagyerdei körút 98. Hungary;* [2]*Department of Microbiology and Biotechnology, Faculty of Natural Sciences, University of Debrecen, H-4010 Debrecen, Egyetem tér 1. Hungary;* [3]*Department of Biochemistry, Leiden University, Gorlaeus Laboratory, P.O. Box 9502 2300 RA Leiden, The Netherlands (*Author for correspondence; E-mail: sbiro@jaguar.date.hu)*

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Abstract

Factor C is an extracellular signal protein involved in cellular differentiation in *Streptomyces griseus*. Nuclease S1 mapping experiments revealed that transcription of the gene takes place from a single promoter in a developmental-stage specific manner. The latter was also confirmed by *in vivo* promoter probing. The sequence of its promoter suggests that the gene is not transcribed by the major sigma factor. The cloned gene expressed from its own promoter in low- and high-copy-number vectors restored normal sporulation to a bald mutant of *Streptomyces griseus*. Computer analysis of the amino acid sequence revealed the presence of a transmembrane localization segment with the N-terminus positioned inside the cell. These data fit well into our working model that points at an important role for factor C in the morphogenesis of *Streptomyces griseus.*

Introduction

The study of the Gram-positive streptomycetes is particularly attractive because of their mycelial life cycle which finally results in sporulation (Chater 1989, 1998) and the concomitant production of secondary metabolites that includes many compounds of great medical and/or industrial importance (Strohl 1997).

Most studies on morphological differentiation and its intimate connection to secondary metabolism have been done in *Streptomyces coelicolor,* the prototype and genetically best characterized *Streptomyces* strain, on solid medium (Chater 1998; Hopwood 1999). However, the industrially important antibiotics are produced by strains other than *Streptomyces coelicolor*, justifying the study of differentiation in other species particularly in submerged culture (Strohl, 1997).

Extracellular regulatory molecules – also called autoregulators – play a key role in controlling cellular differentiation and secondary metabolism in streptomycetes. One group is the low molecular weight γ-butyrolactone A-factor and related compounds. A-factor controls both aerial mycelium formation and antibiotic biosynthesis in *S. griseus* (Khokhlov 1991). Detailed studies on the biosynthesis and on the genetics of its mode of action have been published (Horinouchi & Beppu 1992; Horinouchi 1996). Closely related compounds in other *Streptomyces* species have also been studied (Yamada et al. 1997).

Another autoregulator of cellular differentiation in streptomycetes is the regulatory protein factor C, an extracellular signal protein that plays a key role in cellular communication and cytodifferentiation. Factor C was isolated from the culture fluid of *S. griseus* 45H which readily sporulates in liquid culture (Szabó et al. 1962; Biró et al. 1980). Our previous results on the effects of the factor C protein have been summarized and the cloning, sequencing and partial analysis of its gene (designated *facC*) were published recently (Birkó et al. 1999; Szabó et al. 1999). The present paper provides a transcriptional analysis of the factor C gene as well as further evidence of its role in regulation of sporulation.

Materials and methods

Strains and culturing conditions

E. coli strains JM109 (Messing et al. 1981) and ET12567 (MacNeill et al. 1992) were used for routine cloning and plasmid propagation. The *S. griseus* strains were *S. griseus* 45H and *S. griseus* 52-1 (Vitális et al. 1963; Birkó et al. 1999), *S. griseus* NRRL B-2682 and a spontaneous bald mutant of it, *S. griseus* B-2682bld (A. Penyige, unpubl.). *S. coelicolor* M145 and its derivative M512 (Δ*redD*Δ*actII*-ORF4; Floriano & Bibb 1996) were obtained from the John Innes Centre strain collection. M512 was used for promoter-probe experiments with pIJ2587.

Protoplast preparation and transformation were performed as described by Hopwood et al. (1985). Soya flour mannitol (SFM) medium (Floriano & Bibb 1996) was used to make spore suspensions, R2YE (Hopwood et al. 1985) was used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants. For liquid culturing of *Streptomyces* YEME (Hopwood et al. 1985), tryptone soy broth (Difco) containing 10% (w/v) sucrose (TSBS), or standard minimal medium (MM; Hopwood et al. 1985) with 1% (w/v) mannitol as carbon source were used. For nutritional shift-down, *S. griseus* 45H was grown in TSBS to an OD_{550} of 0.7, washed and transferred to MM.

DNA sequence

The sequence of the *facC* gene was published previously (Birkó et al. 1999). The GenBank accession number is AF103943.

Plasmids and facC *constructs*

Plasmids pIJ2925 (a derivative of pUC19 with *Bgl*II sites at either side of the polycloning site; Janssen & Bibb 1993), pBluescript II KS+ (Stratagene), pHJL401 (Larson & Herschberger 1986) and pWHM3 (Vara et al. 1989) were used for cloning experiments. The *E. coli/Streptomyces* shuttle vectors pHJL401 and pWHM3 were used as low- and high-copy-number vectors in *S. coelicolor* and *S. griseus*, giving 10 and 100 copies per chromosome, respectively.

Plasmid pBZ3 harbours a 2.8 kb DNA fragment including the *facC* gene in pBluescript II KS+ (Birkó et al. 1999). The plasmid pSGF4 was described earlier, and was used as a low-copy-number vector to complement the lack of *facC* in *S. griseus* 52-1 (Birkó et al. 1999). It contains a 2.1 kb fragment encompassing the complete coding sequence of *facC* as well as approximately 1000 bp of upstream and 200 bp of downstream sequences, cloned in pHJL401 (Figure 1). Cloning of the insert of pSGF4 as a *Kpn*I-*Hin*dIII fragment into pWHM3 resulted in the multi-copy-number vector pSGF5. pSGF7, used for generating PCR fragments for nuclease S1 mapping experiments, was made by cloning of the 547 bp PCR fragment made by oligonucleotides fac2 and fac8 (see below and Figure 2) into the *Sma*I site of pIJ2925; in this way a clone with the start of *facC* closest to the *Hin*dIII site of the vector was obtained.

Promoter-probe experiments

The promoter-less *redD* gene present in pIJ2587 (van Wezel et al. 2000) was used as a reporter gene for screening of *in vivo* promoter activity in *S. coelicolor* M145. Cloning of the 550 bp insert of pSGF7, containing the complete intergenic region between *facC* and the upstream located ORF (Figures 1 and 2), into pIJ2587 resulted in pIJ2587-*facC*p. This construct was transformed to *S. coelicolor* M512, using the same strain harbouring pIJ2587 without insert as the control.

Nuclease S1 protection assays and PCR-generated probe

RNA was purified as described by Hopwood et al. (1985), except that DNase I treatment was used in addition to salt precipitation to eliminate DNA from the nucleic acid preparations. For each nuclease S1 protection assay, about 0.02 pmol (approximately 10^4 Cerenkov counts min^{-1}) of labelled probe was hybridised to 30 μg of RNA in NaTCA buffer (Murray 1986) at 45 °C overnight after denaturation at 70 °C for 15 min. All subsequent steps were carried out as described previously (Strauch et al. 1991), using an excess of probe.

The 600 bp probe for mapping *facC* transcripts was generated by PCR amplification using the universal primer (17 mer), the ^{32}P end-labelled fac2 (Figure 1), and pSGF7 as the template. The probe contains an approximately 50 nt non-homologous extension at the 3′ end, to allow discrimination between DNA-RNA hybrids and reannealed probe. PCRs were carried out with 5 u *Pfu* polymerase (Stratagene) in the buffer supplied, and further containing 0.2 mM of each dNTP, 30 pmol of each primer, 10 ng of pSGF7, and 5% DMSO in a total volume of 100 μl. Samples were subjected

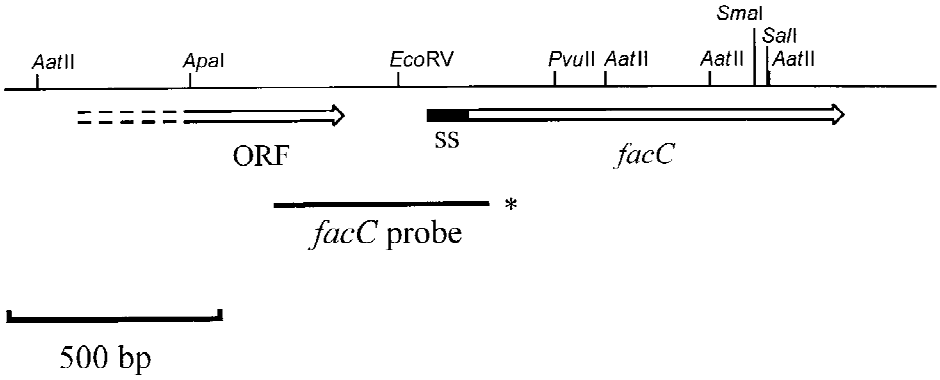

Figure 1. Restriction map of *facC* and flanking sequences (insert of pSGF4). Arrows represent open reading frames. The probe used for S1 mapping is shown; the asterisk marks the position of the ^{32}P-labelled 5′ end. **SS**, secretion signal sequence.

to 30 cycles of: 60 s at 94 °C, 60 s at 54 °C and 60 s at 72 °C.

Southern hybridization

Chromosomal DNA samples from *S. griseus* strains 45H, 52-1, NRRL B-2682 and its spontaneous *bald* mutant *S. griseus* B-2682bld, as well as pBZ3 and pBluescript II KS+ plasmid DNAs were digested to completion with *Sac*II. DNAs were size-fractionated by gel electrophoresis on a 1% (w/v) agarose gel in 1×TAE and transferred to Hybond-N (Amersham) membranes according to the manufacturer's instructions. Hybridization was carried out at 42 °C, in a mixture containing 50% (v/v) formamide, 2×SSC, 5×Denhardt solution, 0.2 mg ml^{-1} denatured salmon sperm DNA and 0.1% (w/v) SDS. For high stringency washing we used a solution of 0.1×SSC + 0.1% (w/v) SDS at 65 °C for 30 min. The 860 bp *Eco*RV-*Sal*I fragment (Birkó et al. 1999), was used as the probe. The DNA was radiolabeled using the Megaprime Kit (Amersham) incorporating [α-^{32}P]dCTP.

Results and discussion

In vivo *promoter probing of the* facC *locus in* S. coelicolor

To determine the presence and approximate location of possible promoters upstream of *facC*, encoding factor C, we used the promoter-probe vector pIJ2587, which contains the promoterless *redD* gene, encoding the transcriptional activator of the biosynthetic genes for the red-pigmented antibiotic (Red) undecylprodigiosin (Narva & Feitelson 1990). Introduction of pIJ2587 containing promoter sequences upstream of *redD*, in *S. coelicolor* M512 (M145 Δ*redD*, Δ*actII*-ORF4) results in production of RedD, and hence activation of the Red cluster. In this way, Red production becomes completely dependent on the promoter inserted in front of *redD* (van Wezel et al. 2000).

A 547 bp DNA fragment was amplified by PCR using oligonucleotides fac2 and fac8 (Figure 2) and cloned into pIJ2587, with the start of *facC* proximal to the *redD* gene, resulting in pIJ2587-*facC*p. Introduction of pIJ2587-*facC*p into *S. coelicolor* M512 led to a low level of Red production on R2YE plates approximately 12 hours after the appearance of aerial mycelium, resulting in pink aerial mycelium 4–5 days after transformation, while control transformants (pIJ2587 without an insert) remained white, with no hint of Red production even after prolonged incubation.

These data suggest that a promoter is present on the 547 bp fragment, most likely in the region upstream of *facC*. Furthermore, regulation of this putative promoter appears differentiation-dependent, such that its activity correlates temporally to a time point just after the onset of aerial mycelium formation. Such a development-dependent regulation fits well with our earlier observation that factor C is essential for morphological differentiation – and particularly for sporulation – of *S. griseus* (Vitális & Szabó 1969; Birkó et al. 1999). Apparently, the sigma factor required for recognition of the *facC* promoter is also present in *S. coelicolor*.

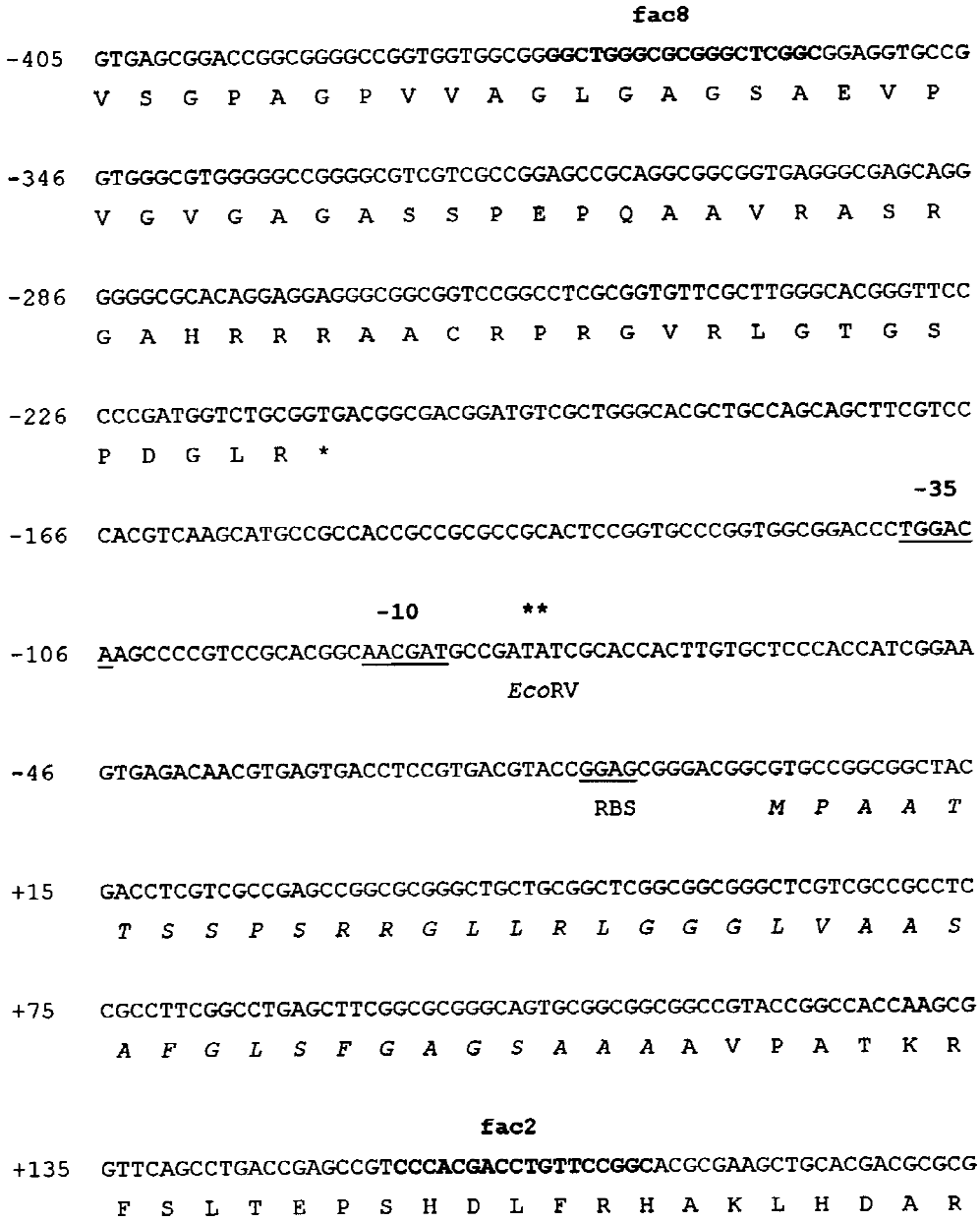

Figure 2. Nucleotide sequence of the start of the *facC* gene and upstream region, including the end of a hypothetical ORF. Amino acid translations of *facC* and the upstream ORF are shown below the sequence; the leader peptide of factor C is italicised. Nucleotide numbers refer to the start of the *facC* gene (first nucleotide = +1). Oligonucleotides fac8 and fac2, used for PCR to generate the insert of construct pSGF7 are shown in bold face. The most likely −35 and −10 sequences are underlined; the asterisks above the DNA sequence refer to the nucleotides representing possible start sites for the transcription of *facC*. RBS, ribosome binding site.

Transcription of facC *takes place from a single promoter*

To analyse transcription of *facC* and to localise its promoter(s), we performed nuclease S1 mapping experiments on RNA isolated from *S. griseus* 45H, using the 600 bp *facC* probe resulting from PCR with ^{32}P-end-labelled oligonucleotide fac2 and the universal primer on pSGF7 DNA. The location of the probe is shown in Figure 1.

We failed to identify transcripts when the RNA used was isolated from cultures grown in liquid TSBS, while a weak band of approximately 245 nt was observed with samples isolated from liquid mm (data not shown). This band corresponded to a position approximately 75 nt upstream of the start of *facC* and, as discussed below, is the transcriptional start site of the *facC* mRNA. Since promoter-probe experiments indicated that transcription of *facC* may be timed in a later stage of *Streptomyces* development, we subjected cultures of *S. griseus* 45H to a nutritional shift-down experiment, which elicits submerged sporulation by this strain. Cultures of *S. griseus* 45H were allowed to grow in TSBS until an OD_{550} of 0.7, washed in MM, and transferred to MM with mannitol as the carbon source. Cultures were incubated at 30 °C and RNA isolated after 0, 30, 60, 120, 240 and 420 min. Under the conditions chosen, small club-like thickenings were formed at the tips of the hyphae after approximately 1.5–2 h following nutritional shift-down, while submerged sporulation started some 5 h later. The RNA was analysed by nuclease S1 mapping, again using the *facC* probe.

Interestingly, while at the time just before shift down no transcript could be identified, shortly afterwards a band of approximately 245 nt appeared, corresponding to a transcript initiated from a site 75 nt upstream of the start of *facC*. Since promoter-probe experiments revealed a possible promoter in the region immediately upstream of *facC*, it is likely that this indeed reflects transcription initiation and not processing of a longer transcript. No full-length transcripts were detected suggesting that transcription of *facC* and the upstream ORF are not transcriptionally coupled. Transcript levels increased over time, and were high more than 4 h after shift down, corresponding to the time between the point when formation of spore-like bodies was initiated and emergence of mature spores in the culture fluid. This confirms that factor C is expressed at a time that can be correlated to the onset of sporulation, and fits well in the model that factor C is involved in sporulation and/or spore maturation in both solid- and liquid-grown cultures (Birkó et al. 1999).

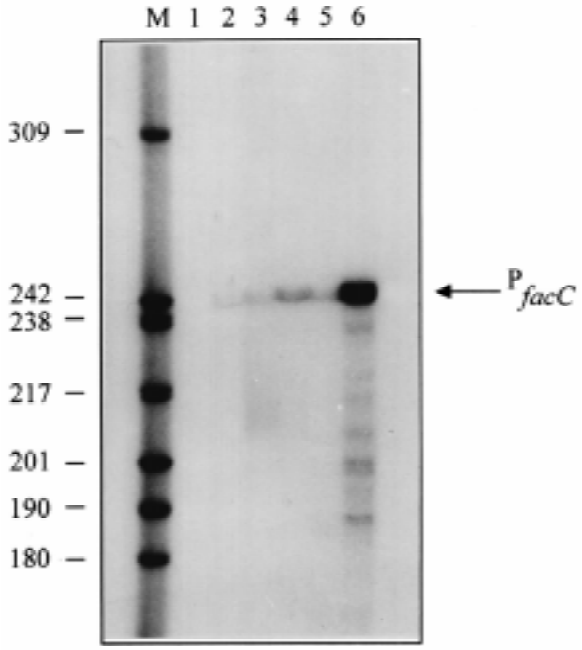

Figure 3. Transcription of *facC* after nutritional shift-down (SD). M, ^{32}P end-labelled *Hpa*II-digested pBR322 size markers; numbers on the left refer to the size of the marker bands (in nucleotides). P_{facC}, *facC* transcript. Lanes 1–6 refer to samples taken 0, 30, 60, 120, 240 and 420 min after SD, respectively. Cultures started to produce spore-like bodies between 60 and 120 min after SD, and mature spores emerged in the culture approximately 400 min after SD.

The transcriptional start site is preceded by the sequences TGGACA around −35 and AACGAT around −10, separated by 17 bp (Figure 2). The A residue at nt position −76 relative to the start of the *facC* gene (Figure 2) constitutes the most likely start of the *facC* transcript since transcription typically starts from a purine residue 6–7 nucleotides downstream of the −10 sequence (Hawley & McClure 1983). The −35 sequence is highly similar to the consensus −35 sequence for major promoters (TTGACA; Hawley & McClure 1983), in *S. coelicolor* recognised by the major sigma factor σ^{hrdB} (Brown et al. 1992). The −10 sequence (AACGAT) shows only low homology to the consensus -10 sequence (TATAAT), although a consensus for *Streptomyces* promoters is difficult to assign (Strohl 1992). However, the timing of *facC* expression during a late stage of development, suggests that the *facC* promoter may not be recognised by the major sigma factor in *S. griseus*.

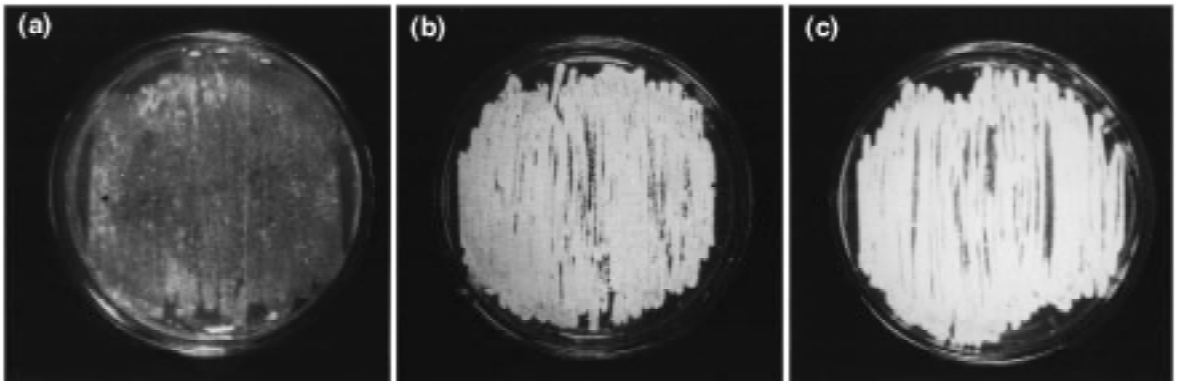

Figure 4. Photographs of 7 days old surface cultures grown on R2YE (Hopwood et al. 1985) of the *bald* mutant *S. griseus* B-2682bld strain (A) and two transformants of it, harbouring the low-copy-number plasmid pSGF4 (B) or the high-copy-number plasmid pSGF5 (C) both expressing *facC* from its natural promoter.

facC *restores sporulation to a* bald *mutant of* S. griseus

A spontaneous bald (*bld*) mutant (*S. griseus* B-2682bld; A. Penyige, unpubl.) of *S. griseus* NRRL B-2682 was transformed with the low-copy-number vector pSGF4 or the high-copy-number vector pSGF5, both harbouring the *facC* gene. As shown in Figure 4, introduction of either construct restored aerial mycelium formation on R2YE plates. Microscopic examination confirmed that spore formation was abundant and by phase-contrast microscopy indistinguishable from that of the wild-type strain (data not shown). *S. griseus* NRRL B-2682 sporulates well in submerged culture. Since the gene for factor C was previously detected in other *Streptomyces* species that sporulate readily in liquid culture, eg. *S. albus* and *S. flavofungini*, we anticipated an important role for factor C in submerged sporulation (Birkó et al. 1999). Restoration of normal development to B-2682bld by introduction of *facC* prompted analysis of its chromosomal DNA, to check whether *facC* was present in this strain. Surprisingly, hybridization of *Sac*II-digested chromosomal DNAs of *S. griseus* NRRL B-2682 and its bald mutant using the facC probe (harbouring most of the *facC* and 75 nt upstream region) not only revealed the absence of the gene in the mutant, but also in the parental strain (Figure 5). Since examples are known (van Wezel, unpubl.) of some DNA preparations in which certain genes may be masked, and detectable only after prolonged proteinase K treatment of the DNA, we made repeated efforts to detect of the gene in NRRL B-2682 and B-2682bld, but unsuccessfully. We also failed to amplify the gene by PCR using primer pairs that were used successfully with DNA of our factor C producer *S. griseus* 45H strain.

How does factor C exert its function?

The data presented in this paper imply that:

(i) Transcription of *facC* takes place from a single promoter located approximately 75 nt upstream of the start of the gene, and is induced by nutritional shift-down, which is widely used to trigger submerged sporulation. Furthermore, promoter probe data suggest that the promoter is transcribed in a differentiation-specific manner. However, these experiments were done in *S. coelicolor* and we cannot ascertain that the sigma factor responsible for transcription of *facC* in *S. griseus* is present in *S. coelicolor*.

(ii) Factor C is not indispensable for submerged sporulation although addition of the protein to the culture fluid was shown to induce this phenomenon in several *S. griseus* strains.

(iii) Despite the absence of *facC*, responsive elements of its signal transduction pathway are present in *S. griseus* NRRL B-2682 and its bald mutant B-2682bld.

(iv) Restoration of aerial mycelium formation to *S. griseus* B-2682bld might be explained by a mutation in a putative functional (but not sequence) homologue of factor C, which like factor C functions as an upstream regulator in the signal transduction pathway. Alternatively, factor C may be a suppressor of the sporulation defect, for example by inducing silent sporulation genes. Both possibilities imply the presence of a complex regulatory network consisting of multiple signal transfer

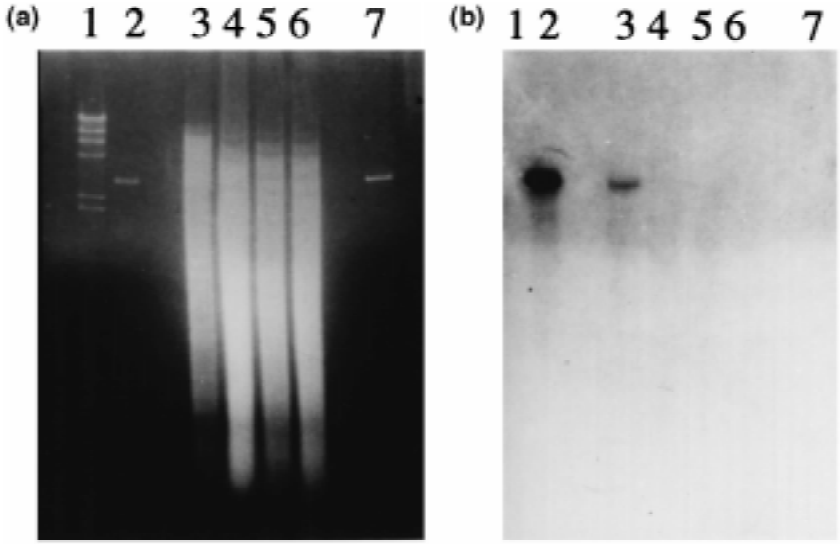

Figure 5. Agarose gel electrophoresis of chromosomal DNAs of different *S. griseus* strains and control plasmid digested with *Sac*II (A) and the autoradiogram of its Southern blot hybridization (B). Lane 1, molecular weight size markers (*Hind*III digested λ DNA); lane 2, pBZ3 (positive control); lane 3, *S. griseus* 45H; lane 4, *S, griseus* 52-1; lane 5, *S. griseus* NRRL B-2682; lane 6, *S. griseus* B-2682bld (bald mutant); lane 7, pBluescript II KS+ (negative control). The 860 bp *Eco*RV-*Sal*I fragment harbouring most of the *facC* gene (Birkó et al. 1999), was used as the probe.

systems acting independently or having complex interactions. Similar results have been reported by several authors (Babcock & Kendrick 1988; Ueda et al. 1993; Kudo et al. 1995).

(v) Factor C may also play a role in morphological differentiation on solid-grown cultures of *S. griseus*. For example, factor C complements a bald mutant, and we therefore anticipate a role early in development. The promoter probe experiment showed expression of the *facC* promoter in aerial mycelium, but we certainly can not rule out the possibility that low levels of factor C (0.5 ng ml^{-1} is already enough to trigger differentiation) are produced in the vegetative hyphae. To study its role in development we are currently attempting to create a *facC* null mutant of *S. griseus* 45H to investigate this point.

Earlier data suggested that factor C is an extracellular signal molecule involved in the regulation of cytodifferentiation. For example, the protein was purified from the culture fluid of *S. griseus* 45H. Furthermore, addition of factor C at concentrations as low as 0.5 ng ml^{-1} to the culture fluid of the responsive strain *S. griseus* 52-1 (that does not produce factor C and is blocked in submerged sporulation) induces the formation of spore-like bodies similarly to that observed in the factor C producer that sporulates well in submerged culture (Szabó et al. 1962; Birkó et al. 1999). The effect of factor C is developmental stage-specific. It exerts its effect only if it is added to the culture between the time of inoculation and 16 h of cultivation. The added factor C rapidly disappears from the culture fluid, probably taken up by the cells (Szeszák et al. 1991). As was predicted by computer analysis of the gene, we showed by amino acid sequence analysis (Birkó et al. 1999; Szabó et al. 1999) of the secreted factor C that it has a signal sequence, which is cleaved off during secretion. Analysis of possible transmembrane regions in factor C and their orientation using several programs including Tmpred (Hofmann & Stoffel 1993) and DAS (Cserzo et al. 1997) suggested the likely presence of a transmembrane domain in factor C, with its N-terminus positioned inside the cell and its C-terminus facing outward. The transmembrane segment extends from amino acid positions 69 to 90 in the mature protein. This suggests that when factor C is taken up by the cells, it is actually integrated into the cytoplasmic membrane. We are currently testing this hypothesis by creating site-directed mutants with amino acid replacements disrupting the predicted TM segment, and are attempting to identify possible receptors and/or targets for factor C.

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