Identification of DNA-binding proteins involved in regulation of expression of the *Streptomyces aureofaciens* *sigF* gene, which encodes sporulation sigma factor σ^F

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Expression of the *sigF* gene encoding a sporulation-specific sigma factor, σ^F , in *Streptomyces aureofaciens* is restricted only to sporulation. Gel mobility-shift assays using protein fractions from different developmental stages of *S. aureofaciens* revealed two different putative proteins specifically bound to the *sigF* promoter region: a protein (designated RsfA) present in young substrate mycelium, and a protein (designated RsfB) present in the course of sporulation. Based on the characteristic profiles of their appearance during differentiation, RsfA might be a repressor and RsfB an activator of *sigF* expression. The location of a specific binding site of the repressor-like protein (RsfA) was determined by gel mobility-shift assays of promoter deletion fragments and by DNase I footprinting analysis. The binding site mapped from nucleotides –87 to –25 relative to the transcription start point of the *sigF* promoter, and overlapped the –35 promoter region. Given the dependence of *sigF* expression upon *whiH*, the putative sporulation transcription factor WhiH was overproduced in *Escherichia coli* and used in the mobility-shift assays with the *sigF* promoter. However, no specific binding was detected, indicating an indirect dependence of *sigF* upon *whiH*.

Keywords: *Streptomyces aureofaciens*, differentiation, repressor, promoter, sigma factor

INTRODUCTION

Streptomycetes are Gram-positive soil bacteria undergoing an exceptional process of morphological differentiation, phenotypically similar to the sporulation of eukaryotic filamentous fungi. The process is regulated at several levels, and in spite of identification of several regulatory genes important in regulation of the process, the detailed mechanism of the regulation of differentiation in streptomycetes still remains obscure (Chater, 1998).

Developmental processes in higher as well as in lower organisms involve the sequential temporal and spatial expression of regulatory genes, which ensures strictly regulated expression of the genes necessary for the morphology and physiology of differentiated cells. The regulation of gene expression at the transcriptional level

by heterogeneity of σ factors of RNA polymerase belongs to the key mechanisms of regulation of differentiation in several bacteria. In the simplest, but the most studied model of bacterial differentiation, *Bacillus subtilis*, the differentiation, comprising endospore formation, is controlled by successive activation of a cascade of σ factors (Kroos *et al.*, 1999). Two genes encoding σ factors having a function in regulation of sporulation have also been identified in *Streptomyces*. *whiG* of *Streptomyces coelicolor* (Chater *et al.*, 1989) and its functional homologue *rpoZ* in *Streptomyces aureofaciens* (Kormanec *et al.*, 1994) have a role in the initiation of differentiation of aerial hyphae into spore chains. The second gene, *sigF*, is essential in the late stage of differentiation, in spore maturation and pigmentation in both strains (Potůčková *et al.*, 1995; Režuchová *et al.*, 1997).

Differentiation of *S. aureofaciens* has several features in common with that of the genetically best studied *S. coelicolor* strain. However, morphologically there are

Abbreviation: tsp, transcription start point.

some differences between these two strains (Kormanec *et al.*, 1998). Though several regulatory proteins are common, the regulation of differentiation has some specific features in each strain (Kormanec *et al.*, 1994, 1996, 1998, 1999). Expression of the *sigF* gene, encoding the late-sporulation-specific σ^F , is temporally committed to sporulation in both strains (Kelemen *et al.*, 1996; Kormanec *et al.*, 1996). Sun *et al.* (1999) have recently shown that expression of *sigF* is spatially limited to the spore compartment, with no evidence of its expression before sporulation septa are formed in *S. coelicolor*. The dependence of *sigF* expression upon early-sporulation-specific σ factor gene *whiG/rpoZ* in both strains indicated a cascade of activation, similar to that occurring in *B. subtilis* (Kelemen *et al.*, 1996; Kormanec *et al.*, 1996). Moreover, besides *whiG*, *sigF* expression has also been shown to depend upon other five *whi* genes (*whiA*, *B*, *H*, *I* and *J*) that are required for sporulation septum formation in *S. coelicolor* (Kelemen *et al.*, 1996). However, *sigF* dependence upon the σ factor encoded by *whiG/rpoZ* is indirect, and the *sigF* promoter is probably recognized by a holoenzyme of RNA polymerase containing some other σ factor (Kelemen *et al.*, 1996; Kormanec *et al.*, 1996). Since at least three of the *whi* genes (*whiB*, *H* and *I*) encode potential regulatory proteins (Ainsa *et al.*, 1999; Davis & Chater, 1992; Ryding *et al.*, 1998), it seems likely that *sigF* expression might be activated by some of these regulatory proteins to ensure its correct temporal and spatial location in the spore compartment.

In the present study, we have investigated proteins which specifically bind to the *S. aureofaciens sigF* promoter region in the course of differentiation. One such protein (Rsfa), which was present only in young substrate mycelium, was investigated in more detail. Its binding site was determined by DNase I footprinting, and was found to overlap the *sigF* promoter region. Given that *sigF* expression takes place only during sporulation, it seems likely that Rsfa represents a repressor-like protein having a role in inhibition of *sigF* expression in substrate mycelium. *S. aureofaciens* sporulation-specific putative transcription factor WhiH (Kormanec *et al.*, 1999) was overproduced in *Escherichia coli* as a whole protein, and with 6 $\times$ His-tagged N-terminal fusion. However, the protein showed no binding to the *sigF* promoter.

METHODS

Bacterial strains, plasmids and culture conditions. *S. aureofaciens* CCM 3239 wild-type was from the Czechoslovak Collection of Microorganisms, Brno, Czech Republic. *E. coli* XL1Blue (Stratagene) was used as a host, and plasmid pBluescript II SK(+) (Stratagene) was used for *E. coli* cloning experiments. *E. coli* expression plasmids pET28a and pET11c were from Novagen. The overexpression was done in *E. coli* BL21(DE3)pLysS (Novagen). For cell-free extract preparation, 10^8 spores of *S. aureofaciens* were inoculated into 20 ml liquid medium NMP (Hopwood *et al.*, 1985) containing different carbon sources at 0.5% (w/v) final concentration, and cultured at 30 °C to early-exponential phase (14 h), late-exponential phase (20 h) and stationary phase (36 h). For

surface cultures, 10^8 spores of *S. aureofaciens* were spread on sterile cellophane membranes placed on Bennet medium (Horinouchi *et al.*, 1983), and grown for 13 (substrate mycelium), 19 (the beginning of aerial mycelium formation), 36 (aerial mycelium approximately at the time of septation) and 60 h (spore maturation). Conditions for *E. coli* growth and transformation were as described by Ausubel *et al.* (1987).

DNA manipulations. DNA manipulations in *E. coli* were done as described in Ausubel *et al.* (1987), and those in *Streptomyces* according to Hopwood *et al.* (1985). DNA fragments were isolated from agarose gel by the GeneClean technique (BIO 101). Nucleotide sequencing was performed by the chemical method (Maxam & Gilbert, 1980). Site-directed mutagenesis was done by the Chameleon mutagenesis kit from Promega.

Preparation of cell-free extracts. Liquid-grown *S. aureofaciens* mycelium was harvested by centrifugation at 12000 g for 10 min, and washed with ice-cold STE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8). Solid-grown mycelium was scraped from the cellophane. All successive steps were carried out at 4 °C. The mycelium was disrupted by grinding with purified acid-washed sea sand in the presence of liquid nitrogen in a mortar for about 5 min. The mixture was then suspended in binding buffer (12.5 mM Tris pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol), and cell debris was removed by centrifugation for 30 min at 30000 g. The cell-free extracts were stored in aliquots at -70 °C. The extracts were used in gel mobility-shift assays and DNase I footprinting experiments.

Partial purification of the DNA-binding protein Rsfa. *S. aureofaciens* was cultured in NMP medium with 0.5% (w/v) glucose at 30 °C for 14 h. All purification steps were done at 4 °C. The mycelium (20 g) was suspended in 60 ml buffer A (20 mM Tris/HCl pH 8.6, 1 mM EDTA, 20 mM KCl, 12% glycerol, 5 mM mercaptoethanol), and disrupted by sonication on ice (30 min total time, 30 s at amplitude 22 microns and 100 s pause; model Soniprep 150, MSE). Following centrifugation at 15000 g for 30 min, the supernatant was precipitated with ammonium sulfate to 50% (w/v) saturation, and the mixture was gently stirred for 1 h at 4 °C. The precipitate, obtained by centrifugation at 15000 g for 30 min, was dissolved in 12 ml buffer A, and dialysed overnight against the same solution. The sample was applied to a DEAE-cellulose column (25 ml; DE 52 Whatman) equilibrated with buffer A. After the column had been washed with the buffer, proteins were eluted with a linear gradient of KCl from 0 to 1 M in a total volume of 500 ml at a flow rate of 1 ml min⁻¹. Fractions containing DNA-binding activity (24 ml) were pooled and dialysed overnight against buffer A. The dialysed sample was loaded onto a Heparin Sepharose CL-6B column (5 ml; Pharmacia). The column was washed with buffer A, and proteins were eluted with a linear gradient of KCl from 0 to 1 M in a total volume of 100 ml at a flow rate of 0.5 ml min⁻¹. Fractions containing DNA-binding activity (8 ml) were pooled and dialysed overnight against buffer A. The dialysed sample was applied to a MonoQ HR 5/5 FPLC column (Pharmacia) equilibrated with buffer A. Proteins were eluted with a linear gradient (20 ml, 0–0.6 M KCl in buffer A). The active samples were dialysed overnight against buffer A and stored at -70 °C.

Protein analysis. Protein concentrations were determined according to Bradford (1976), with BSA as a standard. Denaturing SDS-PAGE of proteins was done as described by Laemmli (1970), and gels were stained with Coomassie blue R250.

Preparation of radiolabelled DNA fragments. A 253 bp

Gel mobility-shift assay. The assays were performed essentially as described by Ausubel *et al.* (1987). ³²P-labelled DNA fragments (0.2 ng; 5000–10000 c.p.m.) were incubated with cell-free extracts or purified protein for 15 min at 30 °C in 15 µl total volume of the binding buffer, 2 µg sonicated salmon sperm DNA and 4.5 µg BSA. After incubation, protein-bound and free DNA was resolved on a non-denaturing polyacrylamide gel containing 4% acrylamide, 0.05% bisacrylamide and 2.5% glycerol, running (after 1 h prerun at 30 mA and 4 °C) in a high-ionic-strength buffer containing 50 mM

DNase I footprinting. Binding reactions were performed in 30 μ l binding buffer, essentially under the same conditions as for the gel mobility-shift assays with 0.5 ng 32 P-labelled DNA fragments (10000–30000 c.p.m.). After incubation for 15 min at 30 $^{\circ}$ C, 3 μ l DNase I solution [5 U DNase I ml $^{-1}$ (Boehringer Mannheim) in 100 mM MgCl $_2$, 100 mM DTT] was added to the binding reaction. The reaction was incubated for 40 s at 37 $^{\circ}$ C, and stopped by 7.5 μ l DNase I stop buffer (3 M ammonium acetate, 0.25 M EDTA, 0.1 mg tRNA ml $^{-1}$), and extracted with 30 μ l alkaline phenol/chloroform. An aqueous phase was precipitated with 3 vols ethanol. The resulting pellet, after washing with 70 % ethanol and Speed Vac drying, was suspended in 5 μ l Maxam loading buffer [80 % formamide, 1 mM EDTA, 10 mM NaOH, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanole FF]. The DNA fragments were analysed on 6 % DNA sequencing gels together with G + A and T + C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). After electrophoresis, the gels were dried and exposed to an X-ray film.

Fig. 2. Gel mobility-shift assays of a 253 bp DNA fragment containing the *sigF* promoter region from –192 to +61 with respect to *tsp* (Fig. 1) with cell-free extracts from *S. aureofaciens*. (a) Retardation of the ^{32}P -labelled DNA fragment (0.2 ng) by 15 µg cell-free extracts from surface cultures of *S. aureofaciens* in the course of differentiation. *S. aureofaciens* was grown for 13 h (lanes 1 and 5), 19 h (lanes 2 and 6), 36 h (lanes 3 and 7) and 60 h (lanes 4 and 8) on solid Bennet medium (Horinouchi *et al.*, 1983). These times correspond to the different stages of morphological differentiation: 13 h, vegetative substrate mycelium; 19 h, the onset of aerial mycelium formation; 36 h, aerial mycelium approximately at the time of septation; 60 h, spore maturation. Specificity of binding is illustrated by addition of 40 ng of the unlabelled 253 bp *sigF* promoter DNA fragment (lanes 1–4). (b) Retardation of the labelled fragment by increasing amounts of cell-free extracts from surface cultures of *S. aureofaciens* in the course of differentiation. Cells were harvested at the indicated time. Lane 1 contains the labelled fragment only. (c) Retardation of the labelled fragment by 15 µg cell-free extracts from *S. aureofaciens* grown in liquid minimal medium MNP (Hopwood *et al.*, 1985) containing 0.5% (w/v) glucose (lanes 2–4) or mannitol (lanes 5–7) to different growth phases: exponential phase, 14 h (lanes 1 and 5); end of the exponential phase, 20 h (lanes 3 and 6); and stationary phase, 36 h (lanes 4 and 7). Lane 1 contains the labelled fragment only. The arrow indicates the free DNA fragment.

Expression of *whiH* in *E. coli*. The *S. aureofaciens whiH* gene (Kormanec *et al.*, 1999) was mutated to introduce a single *NdeI* site in the start codon using a mutagenic primer, mut87 (5'-CGGCCGACAAAGGACATATGAGTACCTTGCG-3'). The gene was then cloned as a 900 bp *NdeI*–*BamHI* fragment in *E. coli* expression plasmid pET11c (Novagen) cut with the same restriction enzymes, resulting in plasmid pIJ-*whiH*1 for wild-type protein, and as a 900 bp *NdeI*–*HindIII* fragment in plasmid pET28a (Novagen), resulting in plasmid pET-*whiH*1 for the N-terminal His-tagged fusion protein. The DNA sequence of the fusion regions was verified by sequence analysis. The host strain for the pET series of expression plasmids, *E. coli* BL21(DE3)pLysS, transformed with the plasmids was grown in LB medium (Ausubel *et al.*, 1987) containing 30 µg chloramphenicol ml⁻¹ and 100 µg ampicillin ml⁻¹ (for pET11c recombinants) or 40 µg kanamycin ml⁻¹ (for pET28a recombinants) at 24 or 37 °C until OD₆₀₀ 0.5. Expression was induced with 1 mM IPTG. After 3 h (at 37 °C) or 14 h (at 24 °C), the cells were harvested by centrifugation at 12000 *g* for 10 min, and washed with ice-cold STE buffer. The pelleted cells were suspended in the binding buffer and disrupted by sonication. The cell lysates were centrifuged for 30 min at 30000 *g* and the supernatants were stored in aliquots at –70 °C.

Purification of His-tagged *WhiH* protein. *E. coli* BL21(DE3)pLysS harbouring pET-*whiH*1 was grown in LB medium at 24 °C until OD₆₀₀ 0.5. After 14 h induction with 1 mM IPTG, the cells were harvested by centrifugation at 12000 *g* for 10 min, and washed with ice-cold STE buffer. The lysis of cells and native purification of His-tagged *WhiH* protein on His-Tag Bind resin (Novagen) was carried out as directed by the manufacturer. The eluted protein was dialysed

overnight at 4 °C against 100 vols binding buffer, cleared by centrifugation at 30000 *g* for 10 min, and the supernatant was stored in aliquots at –70 °C.

RESULTS

Detection of *sigF* promoter-binding proteins in the course of differentiation by gel mobility-shift assays

Previously, we identified a gene, *sigF*, encoding an alternative σ^F of RNA polymerase that proved to have a role in the late stages of morphological differentiation in *S. aureofaciens* and *S. coelicolor* (Potůčková *et al.*, 1995; Režuchová *et al.*, 1997). The gene was expressed only during sporulation, and it seemed to be indirectly dependent upon early sporulation sigma factor σ^{RpoZ} (Kormanec *et al.*, 1996). Transcriptional control is often mediated by transcriptional factors that bind operator sites. Similarly, regulation of *sigF* expression might include some regulatory proteins activating or repressing *sigF* promoter activity. This could ensure temporal and spatial location of *sigF* expression during differentiation. To search for the presence of such transcriptional regulators, a gel mobility-shift assay was performed with the 253 bp *sigF* promoter fragment (region from –192 to +61 with respect to the *tsp* of the *sigF* promoter fragment; Fig. 1) and cell-free extracts from various developmental stages of *S. aureofaciens* grown on solid medium. Two distinct complexes were found, depending upon the developmental stage. A specifically retarded complex of identical mobility was found with

cell-free extracts from substrate mycelium (13 h), and from the onset of aerial mycelium formation (19 h). A new complex of different mobility was identified during sporulation (36 h), which disappeared later in the process of spore maturation (60 h). The specificity of the interaction was demonstrated by the competitive binding of the unlabelled fragment by these proteins (Fig. 2a). The first complex is stronger (or more DNA-binding protein is present in the cell-free extract), since almost total shift was found with only 10 µg total proteins in the gel mobility-shift assay (Fig. 2b). With both cell-free extracts (from 13 and 19 h), two other weak retarded complexes of lower mobility were visible. These might correspond to a multimeric form of the protein bound in a complex of higher mobility, or some other proteins interacting with the promoter region. We tentatively named the protein that specifically bound the *sigF* promoter region in early stages (13 and 19 h) as RsfA (regulator of *sigE*), and the protein that caused the mobility-shift during sporulation (36 h) as RsfB.

Gel mobility-shift assays with cell-free extracts from *S. aureofaciens* cultured in liquid media

Since *S. aureofaciens* is unable to sporulate in liquid medium, where it grows as substrate mycelium, we performed gel mobility-shift assays with the *sigF* promoter fragment (Fig. 1) and cell-free extracts from *S. aureofaciens* grown in liquid minimal medium NMP with 0.5% (w/v) carbon source. Since some developmental mutants are affected by glucose catabolic repression (Chater, 1998), we used two different carbon sources, glucose and mannitol. Substrate mycelium was harvested at three different growth phases (14 h – early-exponential phase; 20 h – late-exponential phase; and 36 h – stationary phase). A specifically retarded complex of identical mobility was found with cell-free extracts regardless of carbon source used (Fig. 2c). It corresponded to the complex found with cell-free extracts from solid-grown cells after 13 and 19 h (see below). The results of this shift assay indicate that the proposed RsfA protein is present mainly in young substrate mycelium. Its amount decreases in the course of growth, being almost undetectable in stationary phase. However, we can not rule out the possibility that it is present in all stages but that its binding activity decreases during growth.

Location of binding sites by gel mobility-shift assays using *sigF* promoter deletion fragments, and by DNase I footprinting

To map binding sites for presumed RsfA and RsfB proteins within the *sigF* promoter fragment, the 253 bp DNA fragment was digested with restriction endonucleases, and purified fragments (Fig. 1a) were used in gel mobility-shift assays with cell-free extracts from various developmental stages of *S. aureofaciens* grown on solid medium (Fig. 3). Assays with *Apa*LI-digested fragment (–25 to +61) showed that neither RsfA nor RsfB binds the fragment. The assays with *Bst*BI-digested

<i>Apa</i> LI (–25 to +61)			<i>Bst</i> BI (–90 to +61)			Full-length (–192 to +61)		
1	2	3	4	5	6	7	8	9

Fig. 3. Mapping of the binding sites for the presumed RsfA and RsfB proteins within the *sigF* promoter fragment. The 253 bp DNA fragment containing the *sigF* promoter region and a series of fragments trimmed from the 5' end (see Fig. 1) were used in gel mobility-shift assays with 10 µg cell-free extracts from surface cultures of *S. aureofaciens* grown for 13 h (lanes 1, 4 and 7) and 36 h (lanes 2, 5 and 8) on solid Bennet medium. Lanes 3, 6 and 9 represent DNA fragments without cell-free extracts. Lanes: 1–3, the 86 bp *Apa*LI-digested fragment (–25 to +61); 4–6, the 152 bp *Bst*BI-digested fragment (–90 to +61); 7–9, the original 253 bp DNA fragment containing the *sigF* promoter region (–192 to +61).

fragment (–90 to +61) revealed a binding activity of the RsfA protein, but not of RsfB. These results suggested differences in the binding of RsfA and of RsfB. Both proteins bind upstream of at least –25 bp. However, binding of RsfB is completely abolished in the *Bst*BI-digested fragment, and binding of RsfA is almost not affected in this trimmed fragment. Thus it seems that the binding site of RsfA is in the fragment from –25 to –90 bp, and RsfB binds further upstream.

To locate RsfA and RsfB binding sites in the *sigF* promoter region, DNase I footprinting assays were carried out using the same 253 bp *sigF* promoter fragment (Fig. 1) and cell-free extracts from various developmental stages. Using the bottom strand from –192 to +61 bp with respect to the *tsp* of the *sigF*-P2 promoter, we found that the identical region from –87 to –25 bp upstream of the *tsp* was clearly protected with both cell-free extracts from 13 and 19 h, respectively (Fig. 4a). This is in agreement with deletion gel mobility-shift experiments, and suggests that the binding is probably caused by the same protein, RsfA, in both extracts. However, we were unable to locate the binding site of the proposed RsfB protein (present in sporulation), owing to a high endogenous nuclease activity in the 36 h cell-free extract (data not shown). Interestingly, the sequence of the RsfA-protected region includes two tandem repeats (5'-ATGCCC, 5'-CGATAT), and an inverted repeat (5'-ACGGTGC-N4-GCACAGT) overlapping a potential –35 region of the *sigF*-P2 promoter (Kormanec *et al.*, 1996). These sequence motifs might be candidates for the RsfA binding.

Fig. 4. DNase I footprints of *S. aureofaciens* cell-free extracts binding to the ^{32}P -labelled 253 bp *sigF* promoter DNA fragment (0.5 ng) (Fig. 1). The vertical bars indicate the position of the RsfA binding site. The numbering is relative to the tsp of the *sigF*-P2 promoter. Lane 1 is without the cell-free extract. Lanes A and T represent G+A and C+T Maxam-Gilbert sequencing ladders, respectively (Maxam & Gilbert, 1980). (a) DNase I footprints with increasing amounts of cell-free extracts from surface cultures of *S. aureofaciens* in the course of differentiation. Cells were harvested at the indicated time. Lanes 2 through 4 contain 70, 170 and 340 μg cell-free extract. (b) DNase I footprints with increasing amounts of cell-free extracts from *S. aureofaciens* grown for 14 h in liquid minimal medium MNP (Hopwood *et al.*, 1985) containing 0.5% (w/v) glucose or mannitol. Lanes 2 through 4 contain 10, 22 and 45 μg cell-free extract. All binding experiments were performed two to three times with independent sets of cell-free extracts and gave similar results.

To prove that the RsfA protein from the early stages of differentiation (13 and 19 h) is identical to the protein present mainly in young substrate mycelium of *S. aureofaciens* cultivated in liquid minimal medium NMP, we carried out DNase I footprinting assays using the same 253 bp *sigF* promoter fragment (Fig. 1) and cell-free extracts from 14 h cultivation with glucose or mannitol. Regardless of carbon source used, the same region from -87 to -25 bp upstream of the tsp was protected, as in the cell-free extracts from early developmental stages after 13 and 19 h (Fig. 4b). This is also consistent with the shift experiments, where the retarded complex of similar mobility was found with these cell-free extracts. This suggests that the same protein, RsfA, is responsible for the binding in cell-free extracts from liquid- and solid-grown cells.

Purification of RsfA

We tried to purify the RsfA protein from *S. aureofaciens* cultured for 14 h in liquid NMP medium with 0.5% (w/v) glucose by monitoring its binding to the *sigF* promoter fragment. Starting with 20 g mycelia and using the purification strategy described in Methods (ammonium sulfate fractionation, and chromatography

on DEAE-cellulose, Heparin Sepharose and MonoQ FPLC), we found that the protein is extremely unstable, and its stability dramatically decreased with decreasing protein concentration. After the last chromatography step, it was almost inactive (gel-shift signals were visible after long exposure). Thus a substantial stabilization of the protein during the whole purification process is required for successful purification to homogeneity for N-terminal sequencing purposes. These experiments are in progress. However, during all purification steps, the RsfA protein was eluted in a single peak, though with decreasing binding activity. This indicates that the DNA-binding protein responsible for the *sigF* promoter fragment binding (RsfA) is a single protein. Moreover, DNase I footprinting with the fraction after DEAE-cellulose revealed the same protected region as with the cell-free extracts (data not shown).

Overexpression of *whiH* in *E. coli*, and gel-shift assays of purified WhiH with the *sigF* promoter

Given the dependence of *sigF* expression upon *whiH* in *S. coelicolor* (Kelemen *et al.*, 1996), it seems plausible that a similar dependence also exists in *S. aureofaciens*, and this potential transcription factor WhiH might be a

Fig. 5. Overproduction and purification of WhiH in *E. coli*. Samples were analysed by SDS-PAGE (12.5% acrylamide). Lanes 1–4 contain crude extracts from *E. coli* BL21(DE3)pLysS carrying the corresponding plasmid, grown at 37 °C, and induced for 3 h with IPTG. Lanes: 1, pET11c; 2, pIJ-whiH1; 3, pET28a; 4, pET-whiH1. Lanes 5–7 contain samples from His-tagged WhiH protein purification from *E. coli* BL21(DE3)pLysS carrying pET-whiH1, grown at 24 °C, and induced for 14 h with IPTG. Lanes: 5, crude extract from induced cells carrying pET28a; 6, crude extract from induced cells carrying pET-whiH1; 7, soluble fraction from induced cells carrying pET-whiH1; 8, purified His-tagged WhiH protein after Ni²⁺-affinity chromatography. The arrow indicates the location of the WhiH protein. Lane 5, molecular mass markers.

candidate for regulation of *sigF* expression. Moreover, similarly to in *S. coelicolor*, *whiH* expression in *S. aureofaciens* is developmentally regulated, with maximal activity during sporulation (Kormanec *et al.*, 1999). Therefore, WhiH might correspond to the DNA-binding protein RsfB, which caused mobility shift during sporulation. We tried to prove this hypothesis by an investigation of a potential binding of WhiH to the *sigF* promoter. Therefore, the *S. aureofaciens* WhiH was overproduced in *E. coli* using a T7 RNA polymerase expression system. Plasmids pIJ-whiH1 (containing wild-type *whiH*) and pET-whiH1 (with 6 × His-tag fusion) were checked by sequencing, and successfully introduced into host strain *E. coli* BL21(DE3)pLysS. Total protein extracts, before and after induction with IPTG, were examined by SDS-PAGE. As shown in Fig. 5, prominent bands were clearly visible after induction with IPTG at 37 °C in the regions corresponding to the theoretical molecular mass of both WhiH (31.8 kDa) and His-tagged WhiH (34 kDa). After 3 h induction with IPTG at 37 °C, almost all WhiH was found in the insoluble fraction, and 6 × His-WhiH was partially in soluble form. When the culture temperature was decreased to 24 °C, almost all 6 × His-WhiH was in soluble form, and also about half of the WhiH was in the soluble fraction. Therefore, we purified the 6 × His-WhiH protein under native conditions (Fig. 5), and used it for gel mobility-shift assay with the *sigF* promoter fragment (Fig. 1). However, using the same binding conditions as for *S. aureofaciens* cell-free extracts, we did not detect any specific shift of the *sigF* promoter fragment. To check that the 6 × His tag did not influence the binding, we used cell-free extracts from *E. coli*

containing both native WhiH (with plasmid pIJ-whiH1) or 6 × His-tagged WhiH (with plasmid pET-whiH1) for gel mobility-shift assays. Similarly, no shift was found using these cell-free extracts.

DISCUSSION

Developmental processes, such as sporulation, in bacteria require correct temporal and spatial expression of the genes necessary for the morphology and physiology of differentiated cells. This is mainly ensured by activation and inhibition of the key regulatory proteins – σ factors of RNA polymerase. This process involves several levels, from inhibition and activation of transcription by transcriptional repressors and activators, respectively, to inhibition of the action of σ factors by their antagonist, anti-sigma factors. Such orchestration at all regulatory levels ensures the correct developmental process. The σ^F factor of RNA polymerase is one of the key sporulation σ factors in *Streptomyces* (Potůčková *et al.*, 1995; Režuchová *et al.*, 1997). Transcriptional analysis of its gene, *sigF*, in *S. aureofaciens* has revealed that it is expressed only during sporulation, at the time when septation of aerial hyphae occurs, after 36 h growth on solid Bennet medium (Kormanec *et al.*, 1996). By S1-nuclease mapping, we have found two very close tps corresponding to putative promoters *sigF*-P1 and *sigF*-P2. However, the results of primer extension analysis in related strain *S. coelicolor* have revealed only the first tsp, corresponding to *sigF*-P2, though S1-nuclease mapping has revealed both tps also in this strain (Kelemen *et al.*, 1996). Thus it seems that the second tsp corresponding to *sigF*-P1 might arise from

Fig. 6. Comparison of the *S. aureofaciens* (SigFSa) and *S. coelicolor* (SigFSc) *sigF* promoter sequences. Whole intergenic regions from the stop codon of the upstream ORF1 to the initiation codon of *sigF* (both in bold and underlined) were compared. The tps of the *sigF* promoters are indicated by bent arrows. The proposed -10 and -35 boxes of the promoters are in bold and underlined. Inverted repeats are designated by thin arrows. Nucleotides in the *S. aureofaciens sigF* promoter region which were protected from DNase I by RsfA binding have a black background. Numbers correspond to the nucleotide positions and refer to the published sequences of the *S. aureofaciens* and *S. coelicolor sigF* genes (Potůčková et al., 1995).

S1-nuclease over-digestion. Therefore, in the present study we refer only to the *sigF*-P2 promoter. Previous studies have also shown that the *sigF* promoter is dependent upon the early sporulation sigma factor σ^{RpoZ} (Kormanec et al., 1996). However, this dependence is very likely indirect, since the *sigF* promoter did not contain σ^{RpoZ} consensus sequence (Kormanec et al., 1999). Thus the *sigF* promoter is likely recognized by a holoenzyme of RNA polymerase containing another σ factor. This temporal, and probably also spatial, transcriptional activation of *sigF* expression seems to be a key regulatory event in the process of *Streptomyces* sporulation, similar to the activation of prespore-specific σ^{F} in *B. subtilis* (Kroos et al., 1999). It is likely that some transcriptional activators or repressors may participate in this process. To investigate this possibility was the main task of the present paper. The results demonstrate a possible participation of such proteins in the regulation of *sigF* expression in *S. aureofaciens*. Investigation of DNA-binding proteins in the course of differentiation actually revealed the presence of two putative proteins, RsfA and RsfB. While RsfA bound the *sigF* promoter during early stages of differentiation (13 and 19 h), RsfB bound the promoter later during sporulation, approximately at the time of aerial mycelium septation into spores (at 36 h). Considering the results of gel mobility-shift assays with deleted *sigF* promoter fragments and DNase I footprinting, it seems that the DNA binding is caused by two independent proteins, RsfA and RsfB. However, this question would be fully answered after isolation of both proteins, investigation of their binding to the *sigF* promoter, and their N-terminal sequencing. Our preliminary experiments with the isolation of the potential repressor-like protein RsfA to homogeneity failed owing to its high instability in the more purified form. The purification of RsfA for N-terminal sequencing purposes, and cloning of the gene encoding this protein, are in progress.

Given the course of *sigF* expression, it is likely that the binding of RsfA might switch off the promoter in the early stages of differentiation, where its expression is not required. This potential repressor-like function of RsfA

is also indicated by DNase I footprinting analysis, where the protein was found to protect a region that overlaps the proposed -35 region of the *sigF* promoter. At the time of sporulation, probably when sporulation septa are formed, the activation of *sigF* might be ensured by an activator, probably RsfB. For this type of *sigF* transcriptional regulation, it is possible that the *sigF* promoter might be recognized by a holoenzyme of RNA polymerase containing a σ factor that is active during all stages of differentiation, like the principal sigma factor σ^{HrdB} , for instance (Kormanec & Farkašovský, 1993). Interestingly, the putative -10 region of the *sigF*-P2 promoter (TGTGAT) shows limited similarity to the -10 consensus sequence (TAgPuPuT) for σ^{HrdB} -dependent promoters (Strohl, 1992). However, the sequence around the -35 region (with the spacer of 16–18 nt) shows no obvious similarity to the corresponding consensus sequence (TTGACN) of the same class of promoters (Fig. 1b). In *E. coli*, promoters with poor similarity to the consensus in the -35 region are frequently positively controlled by activators that bind upstream of the -35 region, and make specific contacts with the holoenzyme of RNA polymerase, thereby activating the process of transcription in various steps (Gralla, 1990). Thus the holoenzyme of RNA polymerase containing σ^{HrdB} might be a candidate for directing the *sigF* promoter. Of course, to prove this assumption needs further experimentation.

Based on the developmental regulation of *whiH* expression in *S. aureofaciens* (Kormanec et al., 1999), and assuming that *S. aureofaciens sigF* expression is dependent upon sporulation-specific transcription factor WhiH, as found in *S. coelicolor* (Kelemen et al., 1996), it raises the possibility that the putative RsfB could be WhiH. However, the results of the binding experiments with the overproduced WhiH in *E. coli* (with or without N-terminal fusion with $6 \times \text{His}$ tag) indicate that this dependence is probably indirect. However, we can not rule out that the failure of *E. coli*-overproduced WhiH binding was caused by an absence of some WhiH modification or an effector molecule, present in *S. aureofaciens*, but absent in *E. coli*.

Summarizing all these results, we suggest a proposed model of regulation of *sigF* in *S. aureofaciens*. During differentiation on solid medium, the putative repressor RsfA binds to the *sigF* promoter and hinders the access of RNA polymerase to the promoter. This binding is relieved by an unknown mechanism after the septation of aerial hyphae into spore compartments. At that time, the putative activator RsfB is synthesized or activated, and binds to the *sigF* promoter, which activates transcription by RNA polymerase holoenzyme containing the σ factor that is present in all stages of differentiation (probably the principal σ^{HrdB} factor). This ensures the temporal and compartment-specific expression of *sigF* in the spore compartment, where after translation σ^{F} could direct the expression of *sigF*-regulon-encoding proteins required for spore maturation. Of course, this model is preliminary, and the activation of σ^{F} might also involve an anti-sigma factor, or other mechanisms, similar to the activation of σ^{F} in *B. subtilis* (Kroos *et al.*, 1999).

Using the gel mobility-shift assay, we also tried to investigate *sigF* expression in the genetically best studied *S. coelicolor* A3(2) strain. However, using cell-free extracts from liquid-grown *S. coelicolor* in the same minimal medium, NMP, no retardation was detected with any extract from different growth phases. Using cell-free extracts from solid-grown cells on minimal MM medium with 0.5% (w/v) mannitol during differentiation, only a weak retarded fragment was identified after 1 d growth (substrate mycelium) (J. Kormanec, unpublished results). Interestingly, the comparison of *sigF* promoters from *S. coelicolor* and *S. aureofaciens* has revealed that, despite the high sequence similarity between these two promoters up to position -58, the *S. coelicolor* *sigF* promoter lacks a large part of the proposed RsfA-protected region (Fig. 6). Thus though regulation of differentiation has several common features between these strains (Kormanec *et al.*, 1994, 1996, 1998, 1999), there are some regulatory circuits that are specific for each strain.

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