Expression of the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase of *Streptomyces aureofaciens* requires GapR, a member of the AraC/XylS family of transcriptional activators

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Expression of the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is developmentally regulated, and induced by glucose in *Streptomyces aureofaciens*. A gene, *gapR*, encoding a protein similar to the AraC/XylS family of bacterial transcriptional regulators was identified upstream of *gap*. The *gapR* gene was constitutively expressed from a single promoter during the course of differentiation. By integrative transformation, via double crossover, a stable null mutant of the *gapR* gene was obtained. The mutation only slightly affected growth, and had no effect on differentiation of *S. aureofaciens*. However, transcription of the GAPDH-encoding *gap* gene was substantially reduced in the *S. aureofaciens* $\Delta gapR$ null mutant, irrespective of carbon source used. Though GAPDH activity was about 1.5-fold lower in the mutant, the substantial enzyme activity remained, suggesting the presence of a second GAPDH which is sufficient to ensure growth. The GapR protein, overproduced in *Escherichia coli*, was shown to bind upstream of the *gap*-P promoter region. The results indicate a direct role of GapR in regulation of *gap* expression in *S. aureofaciens*.

Keywords: glucose induction, differentiation, GAPDH, promoter, S1-nuclease mapping

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12), a key enzyme of glycolysis, plays a crucial role in catabolic and anabolic carbohydrate metabolism. It reversibly catalyses the oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using NAD⁺ as a coenzyme (Harris & Waters, 1976). Although GAPDH-encoding genes have been identified in various bacteria, detailed study of regulation has been done mainly in *Escherichia coli*. It contains two functional *gap* genes, *gapA* and *gapB*. In spite of the fact that the *gapB* gene is located in an operon with the other glycolytic gene, *pgk*, encoding phosphoglycerate kinase, a very low phosphorylating GAPDH activity was measured for this gene product,

and it displayed high nonphosphorylating erythrose-4-phosphate dehydrogenase activity (Alefounder & Perham, 1989; Boschi-Muller *et al.*, 1997). Since a mutation in the *gapA* gene abolished the GAPDH activity, the *gapA* gene was considered to be the only active GAPDH-encoding gene in *E. coli* (Branlant *et al.*, 1983; Della Seta *et al.*, 1997). Analysis of *gapA* transcription revealed a complex regulation comprising four tandemly organized promoters (Charpentier & Branlant, 1994). The *gapB*–*pgk* operon is directed by a single promoter (Charpentier *et al.*, 1998). Interestingly, expression of both *gap* genes is strongly stimulated by the presence of glucose in the medium, and this induction depends upon a component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), the EII^{Glc} glucose-permease protein (Charpentier *et al.*, 1998). Recently, glucose induction of *gapA* expression was also demonstrated in the low-GC Gram-positive bacterium *Bacillus subtilis*. This induction has been shown to depend indirectly on a key protein for catabolite repression, catabolite control protein (CcpA),

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tsp, transcription start point(s); wt, wild-type.

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which belongs to the LacI/GalR transcriptional repressor family (Fillinger *et al.*, 2000; Tobisch *et al.*, 1999).

Streptomyces are mycelial, high-GC Gram-positive bacteria that undergo a complex cycle of morphological differentiation involving the development of spore-bearing aerial hyphae on mycelial colonies. They produce a variety of biologically active secondary metabolites, including the majority of known antibiotics (Chater, 1998). Previously, we identified and characterized the GAPDH-encoding *gap* gene in *Streptomyces aureofaciens* (Kormanec *et al.*, 1995). Transcriptional studies of the *gap* gene suggested monocistronic organization and developmental regulation of the gene. A single promoter, *gap*-P, was induced by the presence of glucose in the medium, and at the onset of aerial mycelium formation (Kormanec *et al.*, 1997). Sequence analysis of the region upstream of *gap* has revealed the 3' end of a gene encoding a protein similar to the AraC/XylS family of bacterial transcriptional regulators (Gallegos *et al.*, 1997; Kormanec *et al.*, 1995). Its close proximity to *gap* suggests a function in the regulation of *gap* expression, since transcriptional regulators of this family are usually located upstream of the operon they regulate (Gallegos *et al.*, 1997). To elucidate a possible function of the gene, which we named *gapR*, in the regulation of *gap* expression in *S. aureofaciens*, we disrupted the gene and analysed expression of the *gap* gene under various conditions in this mutant. The GapR protein, overproduced in an *E. coli* expression system, was shown to bind upstream of the *gap*-P promoter region. The results suggest a direct role of GapR in regulation of *gap* expression in *S. aureofaciens*.

METHODS

Bacterial strains, plasmids and culture conditions. *S. aureofaciens* CCM 3239 wt 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 plasmid pET28a was from Novagen. The overexpression was done in *E. coli* BL21(DE3)pLysS (Novagen). For protoplast preparation and DNA isolation, *S. aureofaciens* was cultured to late-exponential phase (24 h) in liquid TSB medium (Hopwood *et al.*, 1985) containing 1% (w/v) maltose and 0.5% (w/v) glycine as described by Kormanec *et al.* (1993). The phenotype of the *S. aureofaciens* Δ *gapR* mutant was analysed after growth on solid minimal MM medium (Hopwood *et al.*, 1985) and rich Bennet medium (Horinouchi *et al.*, 1983) containing appropriate carbon source at 1% (w/v) final concentration. For RNA isolation from liquid-grown cultures, 5×10^8 c.f.u. of *S. aureofaciens* strains were inoculated in 50 ml liquid medium NMP (Hopwood *et al.*, 1985) containing mannitol or glucose (0.5%, w/v) as a carbon source, and grown at 30 °C to different growth phases: exponential phase, 12 h; end of the exponential phase, 20 h; and stationary phase, 30 h. For RNA isolation from surface culture, 10^8 c.f.u. of *S. aureofaciens* were spread on sterile cellophane membranes placed on Bennet medium (Horinouchi *et al.*, 1983), and grown for 13 h (substrate mycelium), 19 h (the onset of aerial mycelium formation) and 36 h (aerial mycelium approximately at the

time of septation). Conditions for *E. coli* growth and transformation are 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* were according to Hopwood *et al.* (1985). DNA fragments were isolated from agarose gels by binding to a DEAE-paper as recently described (Kormanec, 2000). Nucleotide sequencing was performed by the chemical method (Maxam & Gilbert, 1980). Site-directed mutagenesis was done with a Chameleon mutagenesis kit from Promega.

Disruption of the *gapR* gene. The plasmid used for disruption of *S. aureofaciens gapR* was prepared as follows. Plasmid pRPO7-11C contained the 1170 bp *Bam*HI-*Pst*I fragment (Fig. 1a) bearing the full-length *S. aureofaciens gap* gene (Kormanec *et al.*, 1995) in pBluescript SK(+). This *gap*-bearing insert was removed as a 1170 bp *Xho*I-*Xba*I (blunt-ended) fragment and inserted into *Xho*I- and *Sma*I-digested plasmid pTSR1 (Kormanec *et al.*, 1998) containing the *Streptomyces azureus* *tsr* gene conferring resistance to thiostrepton, to create pRPO7-11S. The region upstream of the *gapR* gene was cloned as a 1250 bp *Eco*RI-*Sma*I fragment (Fig. 1a) into *Eco*RI- and *Sma*I-digested plasmid pAPH11 (Kormanec *et al.*, 1998) containing the kanamycin-resistance gene of Tn5, to create pRPO7-11T. The resulting plasmid, pRPO7-11U, was prepared by inserting a 2400 bp *Xba*I-*Xho*I (blunt-ended) fragment from pRPO7-11S into pRPO7-11T cut with *Xba*I and *Sac*I (blunt-ended). The plasmid pRPO7-11U was used to transform *S. aureofaciens* protoplasts as described by Kormanec *et al.* (1993). Thiostrepton-resistant clones were further analysed for thiostrepton resistance and kanamycin sensitivity, which might indicate a double crossover event. Two kanamycin-sensitive clones were identified, and correct integration was confirmed by Southern blot hybridization. Both clones had a similar phenotype, and one clone, *S. aureofaciens* Δ *gapR*, was chosen for further study.

RNA isolation and S1-nuclease mapping of the *tsp*. Total RNA was prepared from cultures of *S. aureofaciens* as recently described (Kormanec, 2000). The integrity of RNA was indicated by sharp rRNA bands after electrophoresis in agarose containing 2.2 M formaldehyde (Ausubel *et al.*, 1987), and staining in ethidium bromide. High-resolution S1-nuclease mapping was performed according to Kormanec (2000). Samples (40 µg) of RNA (estimated spectrophotometrically) were hybridized to approximately 0.02 pmol DNA probe labelled at one 5' end with [γ -³²P]ATP (approx. 3×10^6 c.p.m. pmol⁻¹). The probes used (Fig. 1a) were: probe 1, a 1000 bp *Age*I-*Bam*HI fragment uniquely labelled on the 5' end at the *Age*I site; probe 2, a 580 bp *Bsi*WI-*Apa*LI fragment uniquely labelled on the 5' end at the *Bsi*WI site. The *hrdB*-P2 promoter probe has been described by Kormanec & Farkašovský (1993). The protected DNA fragments were analysed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980).

Preparation of cell-free extracts. Liquid-grown *S. aureofaciens* mycelium was harvested by centrifugation at 12000 g for 10 min, and washed by ice-cold STE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8). The mycelium was disrupted by sonication on ice (30 min total time, 30 s at amplitude 22 microns and 100 s pause; model Soniprep 150, MSE). Cell debris was then removed by centrifugation for 30 min at 30000 g. The cell-free extracts were stored in aliquots at -70 °C.

Protein analysis. Protein concentrations were determined according to Bradford (1976), with BSA as a standard.

Fig. 1. (a) A 3.6 kb *S. aureofaciens* *TaqI* chromosomal fragment (open box) containing the *gapR* and *gap* genes (black boxes), and chromosomal DNA of *S. aureofaciens* Δ *gapR*, disrupted by a thiostrepton-resistance gene (*tsr*) (hatched box) via double crossover. The positions of the fragments cloned in the plasmids used for *gapR* disruption are shown by stippled boxes. The lines below the maps represent DNA fragments (5'-labelled at the end marked with asterisks) that were used as probes in S1-nuclease mapping. Bent arrows represent the direction of the apparent transcripts from *gapRp* and *gap-P* promoters. The black bars below the map of the wt allele represent DNA fragments (5'-labelled at the either end) that were used for gel mobility-shift assays and DNaseI footprinting. Numbers indicate nucleotide positions relative to the tsp of the *gap-P* promoter. The black bar below the map of the Δ *gapR*-disrupted allele represents the probe (1000 bp *Bam*HI–*Bam*HI fragment from plasmid pRPO7-11U) used for Southern hybridization analysis. Relevant restriction sites are indicated. (b) Southern hybridization analysis of chromosomal DNA from the gene replacement experiments. Lanes: 1, *Bam*HI-digested *S. aureofaciens* wt DNA; 2 and 3, *Bam*HI-digested DNA from the two *S. aureofaciens* Δ *gapR* mutant strains; 4, *Bst*EII-digested *S. aureofaciens* wt DNA; 5 and 6, *Bst*EII-digested DNA from the two *S. aureofaciens* Δ *gapR* mutant strains; 7, *Bst*EII + *Eco*RV-digested *S. aureofaciens* wt DNA; 8 and 9, *Bst*EII + *Eco*RV-digested DNA from the two *S. aureofaciens* Δ *gapR* mutant strains. Lambda DNA-*Bst*EII digest was used as the molecular mass standard.

Denaturing SDS-PAGE of proteins was done as described by Laemmli (1970), and gels were stained with Coomassie blue R250. GAPDH was assayed by the arsenolysis procedure; 1 unit (U) is defined as the amount of enzyme which reduces 1 mmol NAD⁺ min⁻¹ at A₃₄₀ (Byers, 1982).

Preparation of radiolabelled DNA fragments for GapR-binding studies. A 291 bp *gap-P* promoter DNA fragment (positions -290 to +1 bp, in relation to the tsp of the *gap-P* promoter; Fig. 1a) was generated by PCR from plasmid pRPO7-11A (Kormanec *et al.*, 1995). For labelling of a coding strand, a 5'-end-labelled oligonucleotide primer, mut73, internal to *gapR* (5'-CACCGGGTACACGCCGATGC-3') and an unlabelled oligonucleotide, mut75, from the *gap-P* promoter region (5'-CCACCAGGTTCCCCGCTGG-3') were used. For labelling of a noncoding strand, oligonucleotide mut75 was 5'-end-labelled, and mut73 was unlabelled. The oligonucleotides were end-labelled with [γ -³²P]ATP (Amersham; 111 TBq mmol⁻¹) and T4-polynucleotide kinase (Biolabs) as described in Ausubel *et al.* (1987). The labelled fragments were purified by PAGE as described in Kormanec (2000).

Gel mobility-shift assay. The assays were done as described by Ausubel *et al.* (1987). ³²P-labelled DNA fragments (0.5 ng, 5000–10000 c.p.m.) were incubated with cell-free extracts or partially purified GapR for 15 min at 30 °C in 15 μ l total volume of the binding buffer (12.5 mM Tris pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol) with 2 μ g sonicated salmon sperm DNA and 4.5 μ g BSA added. After incubation, protein-bound and free DNA were resolved on nondenaturing polyacrylamide gels (4% acrylamide, 0.05% bisacrylamide and 2.5% glycerol), running in a high-ionic-strength buffer (50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5) at 4 °C. The gels were dried and exposed to an X-ray film.

DNaseI footprinting. Binding reactions were performed under the same conditions as for the gel mobility-shift assays with 4 ng ³²P-labelled DNA fragments (10000–30000 c.p.m.), in 30 μ l binding buffer. After incubation, 3 μ l DNase I solution [5 U DNase I ml⁻¹ (Boehringer Mannheim) in 100 mM MgCl₂, 100 mM DTT] was added to the binding reaction. The reaction mixture was incubated for 40 s at 37 °C, and stopped

Fig. 2. Amino acid sequence alignment of *S. aureofaciens* GapR (GapRsa), *S. griseus* AdpA (AdpAsg) (Ohnishi *et al.*, 1999), two *S. coelicolor* AraC-like proteins (SC8F4.20c, SCGD3.05) (accession nos AL137242 and AL096822, respectively) and *E. coli* AraC (AraCEc) (accession no. SP:P03021). The positions of two α -helix-turn- α -helix (HTH) DNA-binding motifs are denoted by arrows above the sequence. Identical residues are highlighted in black. Similar residues are shaded. The numbers indicate amino acid positions.

with 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. The 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.

Expression of gapR in *E. coli*. The *S. aureofaciens* gapR gene was mutated to introduce a single NdeI site in the translational start codon using a mutagenic primer, mut79 (5'-GT-ACGAGGTACATATGTGCGGCGCGG-3'). To produce N-terminal His-tagged fusion GapR protein, the gene was cloned as a 1200 bp NdeI-SacI fragment in *E. coli* expression plasmid pET28a (Novagen) cut with the same enzymes, resulting in plasmid pET-gapR1. The DNA sequence of the fusion region was verified. The host strain for the pET series expression plasmids, *E. coli* BL21(DE3) pLysS, transformed with the plasmid, was grown in LB medium (Ausubel *et al.*, 1987) containing 30 μ g chloramphenicol ml⁻¹ and 40 μ g kanamycin ml⁻¹ at 30 °C until OD₆₀₀ 0.5. Expression was induced with 1 mM IPTG. After 3 h, 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. The purification of His-tagged GapR protein on His-Tag Bind resin (Novagen) under denaturing conditions was carried out as directed by the manufacturer.

Partial purification of GapR. *E. coli* cells containing pET-gapR1 (2 g) were suspended in 10 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. Following centrifugation at 15000 g for 30 min, the supernatant 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, concentrated and dialysed overnight against binding buffer. The sample was stored at -70 °C.

RESULTS

Cloning and sequencing of the gapR gene

We previously identified the *S. aureofaciens* gap gene encoding GAPDH. Sequence analysis of the region upstream of gap revealed the 3' end of an incomplete gene encoding a protein similar to the AraC/XylS family of bacterial transcriptional regulators (Kormanec *et al.*, 1995). In order to clone the complete gene, an *S. aureofaciens* CCM 3239 genomic library (2-4 kb *TaqI* partially digested chromosomal fragments cloned into the *Clal* sites of pBR322) was hybridized with a 380 bp *TaqI*-*Bam*HI DNA fragment upstream of the gap gene (Kormanec *et al.*, 1995). Two positive clones containing overlapping DNA fragments were identified, and the region 1272 bp upstream of gap was sequenced on both strands (Fig. 1a). Colinearity of the cloned DNA fragment with the *S. aureofaciens* chromosome was proved by Southern blot hybridization (data not shown). Analysis of the sequence by the program CODON

Fig. 3. High-resolution S1-nuclease mapping of the tsp for promoters directing expression of the *S. aureofaciens* *gapR* and *gap* genes. (a) Identification of the tsp for the *gapRp* promoter using probe 1 (Fig. 1a) in the *S. aureofaciens* wt strain. (b) Identification of the tsp for the *gap-P* promoter (Kormanec *et al.*, 1997) using probe 2 (Fig. 1a) in the *S. aureofaciens* wt and Δ *gapR* mutant strains. (c) Control S1-nuclease mapping experiment with the same RNA samples using a DNA probe for the *hrdB-P2* promoter (Kormanec & Farkašovsky, 1993). Lanes A and T are G+A and T+C Maxam and Gilbert sequencing ladders (Maxam & Gilbert, 1980). The lane numbers correspond to the time points at which mycelium was harvested for RNA isolation from cultures grown in liquid or solid media (see Methods). Lane C corresponds to *E. coli* tRNA used as a control in S1-nuclease mapping experiments. All S1-nuclease mapping experiments were performed twice using independent sets of RNA with similar results. In every experiment, the same RNA preparations were hybridized in parallel with both probes.

PREFERENCE (Wright & Bibb, 1992) showed that the complete gene had a codon usage typical for *Streptomyces* genes (Wright & Bibb, 1992). There are several in-frame potential start codons. The GTG codon at position 325 was considered the most likely start codon since it is preceded by a sequence (GGAGGT) showing similarity to a RBS (Baylis & Bibb, 1987). The gene encodes a deduced protein of 278 aa and predicted molecular mass of 30014 Da. Based on its location and role in *gap* regulation (see below), we named the gene *gapR*. Comparison of the deduced GapR protein with databases revealed extensive sequence similarity to all members of the AraC/XylS family of transcriptional regulators, especially in the C-terminal DNA-binding domain (Gallegos *et al.*, 1997). The *S. aureofaciens* GapR contains almost all residues of the AraC/XylS family signature motif (Gallegos *et al.*, 1997). Like other members of the family, GapR was found to contain two α -helix–turn– α -helix DNA-binding motifs in the C-terminal region, at positions equivalent to those found in AraC (Fig. 2). Almost all members of the AraC/XylS family contain a conserved C-terminal DNA-binding domain, and nonconserved N-terminal domain that is presumed to contain binding sites for activator molecules that confer specificity (Gallegos *et al.*, 1997). It is striking that GapR is similar to two homologues of AraC/XylS predicted by the *Streptomyces coelicolor* genome sequencing project (www.sanger.ac.uk/Projects/S_coelicolor/) and to the *Streptomyces griseus* A-factor-responsive transcriptional activator AdpA involved in streptomycin biosynthesis (Ohnishi *et al.*, 1999) in this nonconserved N-terminal region (Fig. 2). The highest overall similarity was to the AraC/XylS homologue SC8F4.20c of *S. coelicolor* (48.5% identical residues).

Transcriptional analysis of the *gapR* gene

To examine the expression of *gapR*, high-resolution S1-nuclease mapping was performed using RNA isolated from surface-grown culture of *S. aureofaciens* during differentiation, and from a liquid-grown strain in the presence of glucose or mannitol as a carbon source. The 5'-labelled probe used for *gapR* is indicated in Fig. 1(a). A single RNA-protected fragment was identified with each RNA using probe 1 (Fig. 3a). The fragment corresponded to a putative promoter termed *gapRp* with a tsp at C (position 204), 120 bp upstream from the most likely translation initiation codon of *gapR* (Fig. 4a). The intensity of the fragment was comparable in RNA samples from cells grown on solid medium (Fig. 3a), suggesting a constitutive expression of *gapR* through differentiation. However, the level of RNA slightly increased at the end of exponential phase and in the presence of glucose in the cells grown in liquid medium (Fig. 3a). The –10 (TACGCT) and –35 (TAAACA) regions of the *gapRp* promoter partially match the consensus sequence of *Streptomyces* promoters recognized by a holoenzyme containing the principal σ factor HrdB (Kang *et al.*, 1997). Moreover, the *gapRp*-promoter-bearing DNA fragment was posi-

Disruption of the *gapR* gene in *S. aureofaciens*

the wt strain. The production of chlorotetracycline (Kormanec *et al.*, 1993) was also not affected by the disruption. However, when grown in liquid minimal medium NMP (Hopwood *et al.*, 1985), the growth rate of the $\Delta gapR$ strain was partially decreased compared to the wt strain. At all time points after about 12 h post-inoculation, the dry weights of the mycelium from *S. aureofaciens* $\Delta gapR$ were about 20 % lower than those observed for the wt strain (data not shown).

We have previously shown that a single *gap*-P promoter directing transcription of *gap* is induced both by glucose, and at the onset of aerial mycelium formation (Kormanec *et al.*, 1997). To investigate whether *gapR* disruption has an effect on *gap* transcription, S1-nuclease mapping was performed using RNA isolated from wt and $\Delta gapR$ *S. aureofaciens* strains during differentiation on solid medium, and grown in liquid minimal medium NMP with mannitol or glucose as a carbon source to different growth phases (Fig. 3). As shown in Fig. 3(b), a single RNA-protected fragment corresponding to the *gap*-P promoter (Kormanec *et al.*, 1997) was identified using probe 2 with the RNA isolated from the wt strain. The level of *gap* mRNA was substantially increased in the wt with glucose in the medium, and at the onset of aerial mycelium formation,

Fig. 5. (a) Overproduction of GapR in *E. coli*. Samples were analysed by SDS-PAGE (12.5% acrylamide). Lanes 1 and 2 contain crude extracts from *E. coli* BL21(DE3)pLysS carrying the corresponding plasmid, grown at 30 °C, and induced for 3 h with IPTG. Lanes: 1, *E. coli* containing pET28a; 2, *E. coli* containing pET-gapR1; 3, purified denatured His-tagged GapR protein after Ni²⁺-affinity chromatography. The arrow indicates the location of the His-tagged GapR protein. Lane 5, molecular mass markers. (b) Gel mobility-shift assays of a 291 bp DNA fragment containing the *gap*-P promoter region (Fig. 1a) with cell-free extracts from *E. coli* overproducing GapR. The ³²P-labelled DNA fragment (0.5 ng) was incubated with 15 µg cell-free extracts from *E. coli* BL21(DE3)pLysS carrying pET28a before induction (lane 2) and 3 h after induction with IPTG (lane 3), and from *E. coli* BL21(DE3)pLysS carrying pET-gapR1 before induction (lane 4) and 3 h after induction with IPTG (lane 5). The specificity of binding is illustrated by addition of 50 ng of the unlabelled 291 bp *gap*-P promoter DNA fragment (lane 6). Lane 1, the labelled fragment only. The arrow indicates the free DNA fragment.

as described previously (Kormanec *et al.*, 1997). However, only a very weak RNA-protected fragment (visible after overexposure of the autoradiogram) was identified with all RNAs from the $\Delta gapR$ mutant strain (Fig. 3b). Its intensity was comparable irrespective of carbon source used or stage of differentiation. The results indicate that the $\Delta gapR$ mutation dramatically affected transcription from the *gap*-P promoter. The level of *gap* mRNA is much lower in the $\Delta gapR$ mutant, irrespective of carbon source or developmental stage. As a control, S1-nuclease mapping was performed with the same RNA samples using a probe fragment specific for the *S. aureofaciens* *hrdB*-P2 promoter, which is expressed fairly constantly during differentiation (Kormanec & Farkašovský, 1993). RNA-protected fragments corresponding to the *hrdB*-P2 promoter were identified with all RNA samples (Fig. 3c). Moreover, a similar pattern of *gap*-P expression to the wt was identified using RNA isolated from the *S. aureofaciens* $\Delta gapR$ mutant strain with the *gapR* gene introduced *in trans* (data not shown).

These results were in contrast with the phenotype of the $\Delta gapR$ mutant strain. GAPDH is an essential glycolytic enzyme for carbon flux in primary metabolism, and such a dramatic decrease of *gap* expression should strongly affect growth of the mutant strain on glycolytic carbon sources. However, the growth rate of the $\Delta gapR$ mutant

was only partially decreased (see above). Therefore, we measured phosphorylating GAPDH activity in cell-free extracts from the wt and $\Delta gapR$ strains grown for 20 h in liquid minimal medium NMP with mannitol or glucose as a carbon source. Similar to results of transcriptional analysis, the specific activity of GAPDH was about 2.5 times higher in the presence of glucose (4.01 ± 0.35 U mg⁻¹) than with mannitol (1.81 ± 0.22 U mg⁻¹) in the wt strain. However, although glucose induction of GAPDH activity was detected in the $\Delta gapR$ strains, the specific activity was decreased (2.6 ± 0.28 U mg⁻¹ with glucose, and 1.17 ± 0.12 U mg⁻¹ with mannitol). The results clearly show that the $\Delta gapR$ mutation also affects GAPDH activity, but *S. aureofaciens* seems to contain another glucose-inducible GAPDH-encoding gene, *gapR*-independent expression of which is sufficient to ensure growth. Our previous hybridization analysis suggested the presence of a second *gap* gene in *S. aureofaciens* (Kormanec *et al.*, 1995).

Overproduction of GapR in *E. coli*, and its binding to the *gap* promoter region

To investigate whether GapR acts directly on *gap*-P induction, we overproduced this protein in *E. coli* and probed its binding in the *gap*-P promoter region. The *S.*

Fig. 6. DNaseI footprints of GapR binding to the 5'-end-labelled 291 bp *gap*-P promoter DNA fragment (4 ng) (Fig. 1a). The vertical bars indicate the position of the GapR-binding site. The numbering is relative to the *tsp* of the *gap*-P promoter. Lane 1 is without the GapR protein sample. Lanes 2 and 3 contain 200 and 400 μ g, respectively, of the His-tagged GapR protein, partially purified by DEAE-cellulose chromatography as described in Methods. Lanes A and T represent G+A and C+T Maxam-Gilbert sequencing ladders, respectively (Maxam & Gilbert, 1980). All binding experiments were performed two times with independent sets of protein samples, and gave similar results.

aureofaciens GapR was overproduced as an N-terminal fusion with the 6 $\times$ His tag in *E. coli* using a T7 RNA polymerase expression system. Total protein extracts of *E. coli* transformed with the plasmid pET-gapR1 and the cloning plasmid pET28a, before and after induction with IPTG at 30 $^{\circ}$ C, were examined by SDS-PAGE. A

prominent band with increasing intensity (with a maximum after 3 h) was clearly visible after induction with IPTG in the region corresponding to a molecular mass of 32 kDa (Fig. 5a). This value corresponds to the calculated M_r of the 6 $\times$ His-tagged GapR protein. However, almost all His-tagged GapR protein was found in the insoluble fraction. The amount of the soluble form did not significantly increase at lower temperature, or after coexpression with *groEL*, *groES* or *trx* genes (data not shown). We also failed to renature the 6 $\times$ His-tagged GapR protein isolated under denaturing conditions by Ni^{2+} -affinity chromatography, employing various conditions. This insolubility is a typical characteristic of regulators within the AraC/XylS family and has hampered the biochemical analysis of these proteins (Gallegos *et al.*, 1997). However, when soluble cell-free protein extracts of *E. coli* transformed with plasmid pET-gapR1 and pET28a, respectively, were used in a gel retardation assay with the ^{32}P -labelled 291 bp *gap*-P promoter DNA fragment (positions -290 to +1 bp, in relation to the *tsp* of the *gap*-P promoter; Fig. 1a), a retarded band was clearly visible only with the cell-free extracts of *E. coli* containing plasmid pET-gapR1 (Fig. 5b). The specificity of the interaction was demonstrated by the competitive binding of the unlabelled fragment (Fig. 5b, lane 6). These results indicate that a small residual portion of GapR is in a soluble form and is capable of binding to the *gap*-P promoter region. However, we were unable to purify it by Ni^{2+} -affinity chromatography under native conditions.

To map the binding site for GapR within the *gap*-P promoter fragment, the ^{32}P -labelled 291 bp *gap*-P promoter DNA fragment was digested with restriction endonucleases, and purified fragments (Fig. 1a) were used in gel mobility-shift assays. Assays with an *Rsr*II-digested fragment (-54 to +1) showed no binding of GapR. Assays with a *Sma*I-digested fragment (-99 to +1) revealed a similar binding activity of GapR protein to that of the whole fragment. To locate this GapR-binding site in the *gap*-P promoter region, DNaseI footprinting assays were carried out using the same 291 bp *gap*-P promoter fragment, 5'-end-labelled at either end. To increase specific activity of GapR, we used GapR protein partially purified by DEAE-cellulose chromatography (as described in Methods). Using the coding strand, we found that the region from -73 to -28 bp upstream of the *tsp* was protected. Using the noncoding strand, we found that the region from -82 to -33 bp was protected (Fig. 6). These results are in agreement with the gel mobility-shift experiments. The position of the binding site is indicated in Fig. 4.

DISCUSSION

A new homologue of the AraC/XylS family of regulatory proteins, GapR, regulates expression of the *gap* gene in *S. aureofaciens*. This conclusion is based on the following experimental data: (i) transcription of the *gap* gene was dramatically reduced in the *S. aureofaciens*

$\Delta gapR$ mutant strain; (ii) *gapR* disruption decreased levels of GAPDH activity; (iii) GapR specifically binds to the *gap* promoter region. Although AraC/XylS homologues (more than 100 are known) are involved in the regulation of various processes (for instance, they control degradation of sugars such as arabinose, cellobiose, melibiose, raffinose, rhamnose and xylose, certain amino acids and alcohols, and they regulate stress response and pathogenesis; Gallegos *et al.*, 1997), *S. aureofaciens* GapR is the first AraC/XylS homologue that is essential for the expression of a gene encoding a glycolytic enzyme.

Recent experiments in Gram-negative *E. coli* and low-GC Gram-positive *B. subtilis* revealed that the expression of *gap* is induced by glucose. However, this induction is caused by different mechanisms in these bacteria. In *E. coli*, glucose induction of both *gap* genes, *gapA* and *gapB*, depends upon a component of the PTS system, the EII^{Glc} protein. However, this dependence is indirect. Glucose is assumed to function as an external signal modulating the phosphorylation state of EII^{Glc}, which transmits the signal to the yet unknown regulator (Charpentier *et al.*, 1998). In *B. subtilis*, two *gap* genes were identified encoding GAPDHs with opposite physiological roles: glycolytic *gapA* and gluconeogenic *gapB*. While *gapA* is induced, *gapB* is strongly repressed by glucose (Fillinger *et al.*, 2000). This glucose induction of the glycolytic *gapA* is indirectly dependent on the catabolite control protein CcpA (Fillinger *et al.*, 2000; Tobisch *et al.*, 1999). The glycolytic *gapA* gene is coexpressed in an operon with the upstream gene *cggR*, which encodes a repressor of the *cggR-gapA* operon. CggR belongs to the SorC/DeoR family of transcriptional regulators. The activity of this repressor is inhibited by glucose, and it is assumed that the role of CcpA in glucose induction is mediated by CggR (Fillinger *et al.*, 2000). Likewise, expression of *gap* is also induced by glucose in high-GC Gram-positive *S. aureofaciens* (Kormanec *et al.*, 1997). However, in contrast to *B. subtilis*, the glycolytic *gap* gene is monocistronic in *S. aureofaciens* (Kormanec *et al.*, 1997), and an activator protein of the AraC/XylS family, GapR, which directly binds to the *gap* promoter region, regulates its expression. Considering S1-nuclease mapping experiments in *S. aureofaciens* wt and $\Delta gapR$ mutant strains, transcription of *gap* is substantially reduced in the $\Delta gapR$ mutant, irrespective of carbon source used for cultivation. Only a very low residual amount of *gap* mRNA was detected in the mutant strain, visible only after overexposure of the gel. Based on these results, it seems that the GapR protein is absolutely required for *gap* transcription. The mechanism of glucose induction in *S. aureofaciens* still remains unclear. However, GapR protein may ensure *gap* expression at an uninduced level, irrespective of the carbon source used. In the presence of glucose, an unknown signal might be transduced to the GapR protein by mechanisms similar to those previously described for *E. coli* or *B. subtilis* (Charpentier *et al.*, 1998; Fillinger *et al.*, 2000). Such modified GapR might ensure induced *gap* expression.

In addition to the glucose induction, the *gap*-P promoter is induced at the onset of aerial mycelium formation (Kormanec *et al.*, 1997), and this induction is also substantially decreased in the $\Delta gapR$ mutant. A possible explanation for this induction might be connected with the phase I glycogen degradation that occurs in the course of *Streptomyces* differentiation in substrate hyphae that undergo aerial mycelium formation (Homerová *et al.*, 1996; Plaskitt & Chater, 1995). Thus, GapR might respond to a common product of glucose and glycogen catabolism that is present in increased amounts either in a high glucose uptake in substrate mycelium grown in the presence of glucose, or after phase I glycogen degradation at the beginning of aerial mycelium formation. It is assumed that glycogen accumulation and degradation may play a role in morphological differentiation (Homerová *et al.*, 1996; Plaskitt & Chater, 1995), and this proposed linking of *gap*-P induction indicates physiological relevance of regulation of glycolysis in connection with differentiation.

Considering the results of the DNaseI footprinting analysis, the proposed binding site of GapR in the *gap*-P promoter spans a region from -73 to -28 bp (for the coding strand), and -82 to -33 bp (for the noncoding strand) (Fig. 4a). This protected region contains a tandem repeat (Fig. 4b) that could be the GapR-binding consensus sequence. Moreover, a similar tandem repeat was identified in the proposed binding site of the *S. griseus* AraC/XylS homologue AdpA, in the *strR* promoter (Ohnishi *et al.*, 1999; Vujaklija *et al.*, 1993) (Fig. 4b). Based on these preliminary binding studies, it is difficult to suggest a consensus element for the GapR-binding site. However, considering the similarity between these two proposed binding sites for GapR and AdpA, this tandem repeat might constitute a consensus sequence of the GapR-binding site. No similarity to any of the other identified binding sites for AraC/XylS transcriptional activators was found, but binding sites for this family are highly variable and, in general, unique for each homologue (Gallegos *et al.*, 1997). The GapR-protected region overlaps the -35 hexamer of the *gap*-P promoter. Thus, surface residues of GapR, like those of many activators, could contact RNA polymerase. This binding is similar to several other activators of AraC/XylS (Gallegos *et al.*, 1997).

Considering the comparison of GapR with other AraC/XylS homologues (Fig. 2), it is conceivable that this type of *gap* regulation also exists in other *Streptomyces* species, since two AraC/XylS-homologous proteins discovered by genome sequencing of *S. coelicolor* (www.sanger.ac.uk/Projects/S.coelicolor/) are very similar to GapR in the N-terminal domain. This domain is generally responsible for an effector binding, and AraC/XylS homologues that are similar in this domain also have similar functions (Gallegos *et al.*, 1997). Searching the nucleotide sequence around these two *S. coelicolor* genes did not reveal any *gap* gene or other glycolytic gene. However, one of these proteins might

activate expression of a *gap* gene located in a different position in the *S. coelicolor* genome. Based on the present data of the genome sequencing project, *S. coelicolor* also contains two *gap* orthologues. However, we did not find any similarity between the *S. aureofaciens gap*-P promoter (including the GapR-binding site) and the regions upstream from the *S. coelicolor gap* orthologues. An intriguing finding was the high sequence similarity between *S. aureofaciens* GapR and AdpA of *S. griseus* (Ohnishi *et al.*, 1999) in this N-terminal region (Fig. 2). The AdpA protein is an A-factor-responsive transcriptional activator of the AraC/XylS family, which binds to the promoter of the pathway-specific regulatory gene *strR* responsible for transcription of streptomycin biosynthetic genes in *S. griseus*. Disruption of *adpA* affected streptomycin biosynthesis and morphological differentiation in *S. griseus* (Ohnishi *et al.*, 1999; Vujaklija *et al.*, 1993). This phenotype is in contrast with the results of *gapR* disruption, which did not affect sporulation and production of secondary metabolites in *S. aureofaciens*. Thus, it seems that these two proteins are not homologous in their function. Moreover, compared to other AraC/XylS homologues, *S. griseus* AdpA has an extraordinary long C-terminal DNA-binding domain (Fig. 2). In this respect, it is interesting that the binding sites of both proteins (GapR and AdpA) are similar (Fig. 4b). This might reflect sequence similarity of these proteins in proposed α -helix-turn- α -helix DNA-binding domains (Fig. 2).

An interesting finding was that, though *gap* transcription was substantially reduced in the $\Delta gapR$ mutant, the mutation only partially affected growth of *S. aureofaciens*, irrespective of carbon source used. Estimation of GAPDH activity actually revealed a decrease of enzyme activity in the $\Delta gapR$ mutant, but the residual activity was still high enough to ensure growth. These results indicate the presence of a second *gap* gene in *S. aureofaciens*, as was also suggested by hybridization studies (Kormanec *et al.*, 1995). Based on the residual GAPDH activity in cell-free extracts in the $\Delta gapR$ mutant, the second *gap* gene seems to be induced by glucose.

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