Transcriptional regulation of the four promoters of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2)

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The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2) is transcribed from four promoters that are recognized by at least three, and probably four, different RNA polymerase holoenzymes, each containing a different σ factor. S₁ nuclease protection studies revealed that transcription from all four promoters is induced by the products of agar hydrolysis and strongly repressed by glucose. Mutants deficient in glucose kinase activity were defective in glucose repression of all four promoters. Mutants were isolated or identified in which transcription from all four promoters had become inducer-independent (i.e. constitutive), establishing the existence of a repressor gene for *dagA* that does not appear to be located within 9 kb of the structural gene. The cloned *dagA* gene was also constitutively expressed in the closely related strain *Streptomyces lividans*, which does not normally make agarase and which presumably lacks the repressor gene. Glucose was still able to repress *dagA* transcription even under conditions of constitutive expression, suggesting that glucose kinase does not mediate its effect via inducer exclusion. Relative differences in the use of the four promoters were not detected during different stages of growth of surface-grown cultures, although *dagA* transcription appeared to peak during the production of aerial mycelium.

Keywords: *Streptomyces coelicolor* A3(2), agarase, *dagA*, glucose repression, glucose kinase

INTRODUCTION

Streptomyces coelicolor A3(2) possesses the unusual ability to utilize agar as sole carbon source (Stanier, 1942; Buchanan & Gibbons, 1974). Agar is a heterogeneous polysaccharide consisting of alternating D-galactose and 3,6-anhydro-L-galactose linked by alternating $\beta 1 \rightarrow 4$ and $\alpha 1 \rightarrow 3$ bonds, with many of the sugar residues replaced by substituted derivatives (Yaphe & Duckworth, 1972). Little is known of the biochemistry of agar utilization in *S. coelicolor* A3(2), but in other agar-utilizing bacteria, e.g. *Pseudomonas atlantica* (Morris *et al.*, 1983) and *Cytophaga*

flavensis (van der Meulen & Harder, 1976), several different enzymes are required. The first stage of agar degradation involves an extracellular diffusible agarase (Dag) which converts the high molecular mass polymer into a series of oligosaccharides, which are likely to be multimers of neoagarobiose (3,6-anhydro-L-galactopyranosyl-(1 $\rightarrow$ 3)-D-galactose). These multimers are substrates for further enzymes involved in agar utilization, which ultimately yield the constituent monomers 3,6-anhydro-L-galactose and D-galactose.

Genetic studies (Hodgson & Chater, 1981) suggested that *S. coelicolor* A3(2) also required several different enzymes for growth on agar. Mutations that impaired agar utilization included lesions in the *aga* locus, which appears to be responsible for further processing or scavenging of agar degradation products (Hopwood *et al.*, 1973; Hodgson & Chater, 1981), and two independent Dag⁻ mutations that mapped some distance from *aga* and that failed to segregate from the integrated copy of the SCP1

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Abbreviation: 2-dog, 2-deoxyglucose.

plasmid found in strains of the NF fertility type (Hodgson & Chater, 1981). One of the *Dag*⁻ mutations, *dagA1*, is apparently the result of a deletion that occurred when SCP1 integrated into the chromosome to generate the original NF strain (Kendall & Cullum, 1986). The *dagA1* mutants cannot be cross-fed by *Dag*⁺ strains (Hodgson & Chater, 1981), implying that the function of at least one other gene whose product is cell-bound and essential for agar utilization was lost during the deletion event, which removed about 40 kb of the chromosome (Kieser *et al.*, 1992). While the fate of the 3,6-anhydro-L-galactose is unknown, the inability of *gal* mutants of *S. coelicolor* A3(2) to utilize agar as sole carbon source suggests that all of the utilized carbon derived from agar is incorporated via D-galactose (Hodgson & Chater, 1981).

The agarase gene (*dagA*) has been cloned (Kendall & Cullum, 1984; Bibb *et al.*, 1987) and sequenced (Buttner *et al.*, 1987). It is transcribed from four promoters (p1–p4), identified by S₁ nuclease mapping, *in vitro* transcription analysis, dinucleotide-primed *in vitro* transcription studies and promoter-probing experiments (Buttner *et al.*, 1987). Remarkably, the four promoters are recognized by at least three, and probably four, different RNA polymerase holoenzymes, each containing a different σ factor (Buttner *et al.*, 1988; Brown *et al.*, 1992). The production of agarase appears to be induced by the products of partial agar hydrolysis (Bibb *et al.*, 1987) and is strongly repressed by glucose (Hodgson, 1982; Bibb *et al.*, 1987; Angell *et al.*, 1992), which also represses the expression of several other genes involved in the utilization of alternative carbon sources by *S. coelicolor* A3(2) (Angell *et al.*, 1992 and references therein). The apparent lack of PEP-dependent sugar phosphotransferase systems in *Streptomyces violaceoruber* (Sabater *et al.*, 1972) and in *Streptomyces aureofaciens* (Novotná & Hostálek, 1985), and the absence of fluctuations in cAMP levels with changes in carbon source in *S. coelicolor* A3(2) (Hodgson, 1980) and *Streptomyces venezuelae* (Chatterjee & Vining, 1982), suggest that the mechanism of glucose repression in streptomycetes is very different from that in *Escherichia coli* (Saier, 1989, 1991). Mutations in the glucose kinase gene (*glkA*) of *S. coelicolor* A3(2) have no effect on glucose transport (Hodgson, 1982), but result in the inability to utilize glucose as carbon source and a pleiotropic loss of glucose repression. Genes that are relieved of glucose repression in *glkA* mutants include *dagA* (Hodgson, 1980, 1982; Angell *et al.*, 1992), genes required for arabinose and glycerol transport (Hodgson, 1982) and for the metabolism of galactose, fructose and glycerol (Hodgson, 1982; Seno & Chater, 1983), and the cloned α -amylase genes of *Streptomyces limosus* (Virolle & Bibb, 1988) and *S. venezuelae* (Virolle *et al.*, 1988). This suggests a key role for *glkA* in mediating glucose repression, in addition to its obligate role in glucose utilization.

The aim of these studies was to determine whether substrate induction and glucose repression of agarase activity occurred at the transcriptional level, how each of the four *dagA* promoters responded to both aspects of regulation, and whether the existence of multiple promoters for *dagA* could be accounted for, at least in part,

by growth phase-dependent differences in the use of the promoters.

METHODS

Bacterial strains and plasmids. See Table 1. All strains were stored as spore suspensions in 20% (v/v) glycerol at -20 °C.

Culture and transformation conditions. Standard media, methods of culture and conditions for plasmid DNA isolation and for transformation were as described by Hopwood *et al.* (1985). The liquid minimal medium was that of Seno & Chater (1983) without PEG-6000. Galactose and glucose were used at final concentrations of 1% (w/v) or 0.5% each when given together. To prepare partially hydrolysed agar, a suspension of 5% (w/v) LabM agar in distilled water was adjusted to pH 2 with 1 M HCl, autoclaved for 12.5 min at 10 p.s.i., and allowed to cool to room temperature before adjusting to pH 7 with 1 M NaOH; it was used at a final concentration of 1% (w/v) when present as sole carbon source or 0.5% when added to cultures containing galactose or glucose. Spore suspensions were thawed, sedimented in a bench centrifuge, washed once with distilled water, recentrifuged and resuspended in 10 ml of Hirsch Complex Medium (Hodgson, 1982) before incubating at 30 °C for 8 h in a 250 ml flask with vigorous shaking. The germinated spores were harvested by centrifugation, resuspended in 5 ml minimal medium lacking a carbon source, and subjected to a 5 s pulse of sonication to disrupt the clumps of aggregated germlings, which were used immediately to inoculate 50 ml of minimal medium to an initial OD₄₅₀ of 0.05–0.1. The cultures were grown at 30 °C with shaking at 300 r.p.m. in 250 ml flasks containing stainless steel springs (Hopwood *et al.*, 1985) until they reached late exponential phase, 12–16 h after inoculation. Minimal medium agar plates contained (g l⁻¹): (NH₄)₂SO₄, 1; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.21; FeSO₄·7H₂O, 0.001. The medium was adjusted to pH 7.2 with NaH₂PO₄ and the relevant carbon source was added to 10 mM after autoclaving.

Genetic techniques. Mutants resistant to 2-deoxyglucose (2-dog) were obtained by spreading spores of *S. coelicolor* A3(2) strain M145 on minimal medium agar plates containing 10 mM arabinose and 100 mM 2-dog (Hodgson, 1982). Protoplast preparation, fusion and regeneration, and the scoring of non-selected markers were as described by Hopwood *et al.* (1985), with 2-dog at 100 mM (Hodgson, 1982). The λ EMBL4 library, a gift of S. J. Gould and D. H. Sherman, was made from M145 DNA partially digested with *Sau*3A1 and size-fractionated; the average insert size was approximately 20 kb.

RNA purification and S₁ nuclease mapping. RNA was purified as described by Hopwood *et al.* (1985), except that DNase I treatment was used instead of salt precipitation to eliminate DNA from the nucleic acid preparations. When RNA was extracted from surface-grown cultures, the mycelium was grown on cellophane discs on the agar surface, scraped from the cellophane and treated directly with cold modified Kirby mix. pIJ4085, a derivative of pIJ2925 (Janssen & Bibb, 1993) with a 1.5 kb *Bam*HI–*Eco*RI fragment that contains the four *dagA* promoters and approximately two-thirds of the *dagA* coding region (Buttner *et al.*, 1987), was used for probe preparation. pIJ4085 was digested with *Ava*II and calf intestinal alkaline phosphatase, and 10 pmol of 5' ends were labelled using polynucleotide kinase and 50 pmol [γ -³²P]ATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹), New England Nuclear] (Sambrook *et al.*, 1989). The labelled DNA was digested with either *Pst*I or *Bam*HI, and the appropriate uniquely end-labelled fragments extracted from a low melting point agarose gel (BRL Ultra Pure). Approximately 50 ng labelled probe was mixed with

Table 1. Bacterial strains and plasmids

Strain	Genotype	Origin and comment
<i>S. coelicolor</i> A3(2)		
M145	SCP1 ⁻ SCP2 ⁻	Hopwood <i>et al.</i> (1985)
L2	SCP1 ⁻ SCP2 ⁻ <i>glkA</i>	Spontaneous 2-dog-resistant derivative of M145
L3	SCP1 ⁻ SCP2 ⁻ <i>glkA</i> Dag ⁺⁺	Spontaneous 2-dog-resistant derivative of M145
L4	SCP1 ⁻ SCP2 ⁻ Dag ⁺⁺	Recombinant from protoplast fusion between L3 and M145
J801	SCP1 ^{NF} SCP2*	Hodgson & Chater (1981)
J1501	<i>hisA1 uraA1 strA1</i> Pgl ⁻ SCP1 ⁻ SCP2 ⁻	Chater <i>et al.</i> (1982)
J1508	<i>hisA1 uraA1 strA1</i> Pgl ⁻ SCP1 ^{NF} SCP2 ⁻	Ikeda <i>et al.</i> (1984)
J1376	<i>hisA1 uraA1 strA1</i> Pgl ⁻ SCP1 ^{NF} SCP2 ⁻ <i>glkAΔ119</i>	Spontaneous 2-dog-resistant derivative of J1508. Ikeda <i>et al.</i> (1984)
<i>S. lividans</i> 66		
TK24	<i>str-6</i> SLP2 ⁻ SLP3 ⁻	Hopwood <i>et al.</i> (1983)
Plasmid		
pIJ513	Derivative of the low copy number plasmid pIJ922 (Lydiat <i>et al.</i> , 1985) with a 3.5 kb <i>EcoRI</i> fragment that contains <i>glkA</i> cloned in the unique <i>EcoRI</i> site of the vector (Ikeda <i>et al.</i> , 1984; Angell <i>et al.</i> , 1992)	
pIJ2014	Derivative of the low copy number plasmid pIJ903 (Lydiat <i>et al.</i> , 1985) with a 5.1 kb <i>BamHI</i> fragment that contains <i>dagA</i> cloned in the unique <i>BamHI</i> site of the vector and with <i>dagA</i> in the opposite orientation to the ampicillin resistance gene of pIJ903 (Angell <i>et al.</i> , 1992)	
pIJ4085	Derivative of pIJ2925 (Janssen & Bibb, 1993) with a 1.5 kb <i>BamHI</i> - <i>EcoRI</i> fragment that contains the four <i>dagA</i> promoters and approximately two-thirds of the <i>dagA</i> coding region (Buttner <i>et al.</i> , 1987)	
pIJ5800	Derivative of pIJ2014 containing <i>dagA</i> and approximately 9 kb of upstream sequence	
pIJ5803	Derivative of pIJ2014 containing <i>dagA</i> and approximately 15 kb of downstream sequence	

50 µg RNA in a small volume of distilled water, lyophilized, and resuspended in 20 µl 3 M sodium trichloroacetic acid hybridization buffer (Murray, 1986; prepared according to Summerton *et al.*, 1983). Following denaturation at 65 °C for 10 min, hybridization was allowed to occur at 45 °C for 14 h, and the samples were treated as described by Hopwood *et al.* (1985). Samples were resuspended in 6 µl of formamide loading dye (Maxam & Gilbert, 1980) and 3 µl were subjected to electrophoresis on a 6% polyacrylamide/7 M urea denaturing gel. Since the sizes of the protected fragments corresponding to the four *dagA* transcripts were known (Buttner *et al.*, 1987), sequencing ladders were omitted and end-labelled fragments of *HpaII*-cut pBR322 were used as size markers instead.

Glucose kinase assays. Mycelium from 50 ml cultures was collected by filtration, resuspended in 3 ml of 50 mM Tris/HCl, pH 7.0, and disrupted on ice by ultrasonic vibration (eight 10 s treatments with 15 s intervals). The sonicates were centrifuged at 7000 r.p.m. (Sorvall SS34 rotor) for 10 min at 4 °C and the supernatants centrifuged again at 70000 r.p.m. (Beckman TL-100 rotor) for 2 h at 4 °C and then stored at -70 °C. Protein concentrations were determined by the method of Bradford

(1976). Glucose kinase assays were as described by Angell *et al.* (1992). Units of enzyme activity were expressed as nmol NADP reduced min⁻¹ (mg total cell protein)⁻¹ at 25 °C. The assays were performed on two independent cultures of each strain.

DNA sequencing. The synthetic oligonucleotides 5' CTAGG-ATCCTCCAGACGAGGGGACGATG 3' and 5' GTAG-GATCCATGGTCGTTCTCTCTTCGATTC 3' were used in the DNA polymerase chain reaction (Erlich, 1989) to amplify 449 bp fragments containing the *dagA* promoter regions from M145 and L3 chromosomal DNA. Each reaction contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% (w/v) gelatin, 100 µM of each of the four dNTPs, 2.5 U *Taq* polymerase, 20 pmol of each primer and 10 ng of chromosomal DNA in a final volume of 100 µl. After denaturation at 95 °C for 5 min, the samples were subjected to 30 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min) and then 10 min at 72 °C. The amplified fragments were cleaved with *BamHI* and cloned in the unique *BamHI* site of the polylinker of M13mp18 (Yanisch-Perron *et al.*, 1985); their sequences were determined using the dideoxy-chain termination method (Bankier & Barrell, 1983; Sanger *et al.*, 1977).

Plaque lifts and Southern hybridization. Plaque lifts and Southern blots were carried out as described by Sambrook *et al.* (1989) and Southern (1975), respectively, using purified DNA fragments containing segments of *dagA* labelled with ^{32}P by nick-translation (Hopwood *et al.*, 1985).

Agarase spot tests. Cultures were inoculated and grown as described above in the presence of either 1% (w/v) galactose or 1% (w/v) galactose plus 0.5% (w/v) partially hydrolysed agar as carbon source. Samples of the culture supernatant were removed 16, 24, 48 and 72 h after inoculation and 20 μl of each was spotted on an agar plate, which was incubated at 30 °C for 24 h. Agarase activity was estimated by measuring the diameter of the clearing zones after staining the plates with 0.05 M iodine in 0.12 M KI.

RESULTS

Substrate induction and glucose repression of *dagA* occurs at the transcriptional level

Growth of *S. coelicolor* A3(2) strain M145 on agar plates containing different carbon sources indicated that galactose is a relatively non-repressing carbon source for agarase production. M145 was subsequently grown in liquid minimal medium supplemented with galactose, glucose or partially hydrolysed agar, and combinations thereof. RNA was isolated from cultures in late exponential phase, and *dagA* transcription assessed by S_1 nuclease protection analysis. Transcription from all four promoters was induced by partially hydrolysed agar, and strongly repressed by glucose [Fig. 1a: the RNA-protected fragments observed in this and subsequent figures that are not marked p1–p4 are believed to result from degradation of larger transcripts; corresponding signals were not observed in *in vitro* run-off transcription experiments (Buttner *et al.*, 1988)]. Since the same RNA preparations gave no indication of degradation when used to protect several other DNA probes from S_1 nuclease digestion (e.g. Caballero *et al.*, 1991), the additional bands presumably reflect the lability of the *dagA* transcripts. Prolonged exposure of the gels to X-ray film revealed low levels of transcription from all four promoters in cultures grown on galactose as sole carbon source (i.e. without partially hydrolysed agar); the levels were even lower in cultures grown in the presence of glucose or glucose plus partially hydrolysed agar (data not shown). When detectable, transcription of *dagA* appeared to occur predominantly from p1 and p4. Addition of partially hydrolysed agar at a final concentration of 0.5% (w/v) to mid-exponential phase cultures grown on galactose as sole carbon source caused a marked increase in the levels of the *dagA* transcripts 2 h after addition; no increase was observed in the absence of the inducer (Fig. 1b; GAL+AGAR 2 HR and GAL 2 HR, respectively).

Mutations in the *glkA* gene of *S. coelicolor* A3(2) result in loss of glucose repression of *dagA* transcription

Glucose repression of agarase production is relieved in *glkA* mutants (Hodgson, 1982). To determine the effect of the loss of glucose kinase activity on glucose repression of *dagA* transcription, putative *glkA* mutants were isolated

from M145 by selecting for 2-dog resistance (Hodgson, 1982). Several of the mutants, which arose at a frequency of about 10^{-5} , could not utilize glucose as sole carbon source in liquid medium (the Glc⁻ phenotype), and the two tested, L2 and L3, lacked detectable glucose kinase activity in an assay that gave values of 116.2 and 85.6 nmol NADP reduced min⁻¹ (mg protein)⁻¹ with extracts from duplicate cultures of M145. Transformation of one of the mutants, L2, with pIJ513 (which carries *glkA*; Ikeda *et al.*, 1984) restored both glucose utilization and 2-dog sensitivity. Thus L2 appears to be a *glkA* mutant. Transcription of *dagA* in L2, and in the parental strain M145, was examined by S_1 nuclease protection analysis of RNA isolated from late exponential phase cultures grown in liquid minimal medium supplemented with galactose, galactose plus partially hydrolysed agar, or galactose plus glucose plus partially hydrolysed agar. Glucose repression of *dagA* transcription was clearly absent in L2, while induction appeared to be unimpaired (Fig. 2a and b). The relatively reduced signal for p3 observed in Fig. 2b (and also in Figs 3 and 4) probably results from preferential degradation of the p3 transcript; p3 transcript levels were generally lower in RNA samples that showed signs of degradation (i.e. that gave RNA-protected fragments that did not correspond to p1–p4).

Isolation of a *dagA* constitutive mutant

L3, another 2-dog-resistant Glc⁻ mutant isolated in the same way as L2, also appeared to be defective in *glkA* since it lacked detectable glucose kinase activity, and both 2-dog sensitivity and glucose utilization were restored by pIJ513. However, colonies of L3 were surrounded by much larger zones of agar clearing than the other 2-dog-resistant mutants, and appeared to be over-producing agarase (the Dag⁺⁺ phenotype). Since DNA amplification occurs frequently in some streptomycetes (Hütter & Eckhardt, 1988), it was possible that over-production reflected an increase in the copy number of *dagA*. However, Southern analysis using pIJ2002 (a pUC18 derivative containing *dagA* on a 5.1 kb *Bam*HI fragment; Buttner *et al.*, 1987) as a nick-translated hybridization probe failed to reveal any difference in the copy number of *dagA* in total DNAs from L3 and M145 that had been digested with either *Bam*HI or *Xho*I (data not shown). S_1 nuclease protection studies of RNA isolated from L3 revealed significant levels of *dagA* transcription in the absence of inducer, although a higher level of transcription from p4, but not from p1–p3, was detected after addition of partially hydrolysed agar (Fig. 2c). Thus *dagA* expression in L3 had become partially inducer-independent (i.e. constitutive). To assess whether this reflected the occurrence of a mutation in the *dagA* promoter region, the DNA polymerase chain reaction was used to amplify and clone 449 bp fragments from both M145 and L3 chromosomal DNA; the fragments contained sequences extending from 233 nt upstream of the p4 transcriptional start site to 2 nt in front of the *dagA* translational initiation codon. Sequencing of both amplified fragments failed to reveal any changes from the published sequence (Buttner *et al.*, 1987).

Fig. 1. S₁ nuclease protection analysis of *dagA* transcription in *S. coelicolor* A3(2) strain M145. (a) Levels of the *dagA* transcripts in RNA samples isolated from late exponential phase cultures grown in the presence of galactose (GAL), glucose (GLU) or partially hydrolysed agar (AGAR), and combinations thereof. (b) Levels of the *dagA* transcripts before (GAL 0 HR), and 2 h after (GAL + AGAR 2 HR) addition of partially hydrolysed agar to mid-exponential phase galactose-grown cultures; levels in a control culture to which partially hydrolysed agar had not been added 2 h after addition of the inducer to the experimental culture are also shown (GAL 2 HR). p1–p4, RNA-protected fragments corresponding to the four *dagA* promoters; SM, end-labelled *Hpa*II-digested pBR322 size markers.

Constitutive expression of *dagA* in L3 might have reflected either a particular allelic form of *glkA*, or the occurrence of an additional mutation at a different locus. To assess the latter possibility, recombinants obtained by protoplast fusion between L3 and J1501 (*hisA1 uraA1 strA1*) were screened for segregation of 2-dog resistance and constitutive *dagA* expression. Since the over-production of agarase on agar plates was likely to result from the same mutation that gave rise to constitutive *dagA* transcription in liquid cultures, the Dag⁺⁺ phenotype was used as an initial screen for potential 2-dog-sensitive,

constitutive *dagA* recombinants. Protoplasts of L3 and J1501 were fused, allowed to regenerate non-selectively and the resulting spores spread on selective media containing arabinose as carbon source. Segregation of the 2-dog^R and Dag⁺⁺ phenotypes was clearly apparent. Only 86 % of the Ura⁺ Str^R 2-dog^R recombinants and 40 % of the His⁺ Str^R 2-dog^R recombinants were Dag⁺⁺. The existence of two independent mutations was confirmed by selecting for Ura⁺ Str^R recombinants, and then testing for inheritance of the 2-dog^R, His⁺ and Dag⁺⁺ phenotypes. Of the Ura⁺ Str^R recombinants tested, 44 % were Dag⁺⁺,

Fig. 2. S₁ nuclease protection analysis of *dagA* transcription in late exponential phase cultures of *S. coelicolor* A3(2) strain M145 (a), and derivatives L2 (b), L3 (c) and L4 (d) grown in the

and of these 84 % were His⁻ 2-dog^S and 16 % were His⁺ 2-dog^R. Of the remaining 56 % of Ura⁺ Str^R recombinants which did not over-produce agarase, 91 % were His⁻ 2-dog^S, 6 % were His⁺ 2-dog^R and 3 % were His⁺ 2-dog^S. 2-dog^R mutants were not observed when similar numbers of spores from a control M145–J1501 protoplast fusion were spread on identical selective media, indicating that the apparent segregation of the 2-dog^R and Dag⁺⁺ phenotypes observed after fusion of the J1501 and L3 protoplasts was unlikely to reflect the occurrence of spontaneous 2-dog-resistant mutants. Furthermore, among the L3–J1501 Ura⁺ Str^R recombinants, only 1.5 % showed segregation of the His and 2-dog phenotypes, consistent with co-inheritance of the closely linked *hisA* and *glkA* loci (Kieser *et al.*, 1992), and the absence of a significant number of spontaneous 2-dog^R mutants.

One of the 2-dog^S Dag⁺⁺ recombinants (L4) was purified and studied further. It showed levels of glucose kinase activity [92.8 and 78.6 nmol NADP reduced min⁻¹ (mg protein)⁻¹ in duplicate cultures] similar to those found in M145, and retained the constitutive phenotype of the 2-dog^R Dag⁺⁺ mutant L3. However, induction of *dagA* transcription by partially hydrolysed agar was strongly repressed by glucose (Fig. 2d), confirming that glucose repression of *dagA* can operate under conditions of constitutive expression.

***dagA* is transcribed constitutively in *S. coelicolor* A3(2) NF strains**

NF strains of *S. coelicolor* A3(2) have lost a 40 kb segment of chromosomal DNA that includes *dagA* (Kieser *et al.*, 1992). To assess if the deletion had removed sequences required for induction of agarase activity, and to confirm the role of *glkA* in glucose repression of *dagA*, J1508 (SCP1^{NF}) and J1376 (SCP1^{NF} *glkA*Δ119) were transformed with pIJ2014, a derivative of the low copy-number plasmid pIJ903 containing the agarase gene, and expression of *dagA* assessed by S₁ nuclease protection studies. Transcription of *dagA* was constitutive in both J1508 and J1376, and glucose repression was observed only in the *glkA*⁺ J1508 (Fig. 3a). Constitutive expression might have resulted from the deletion of sequences flanking *dagA* upon integration of SCP1 during the formation of the original NF strain. Alternatively, the *dagA* fragment cloned in pIJ2014 could have lost sequences essential for induction. Expression of *dagA* was therefore analysed in M145(pIJ2014) and in its NF derivative J801(pIJ2014). Transcription was induced by partially hydrolysed agar in M145(pIJ2014), but was constitutive in J801(pIJ2014) (Fig. 3b). Thus constitutive expression was not associated with either pIJ2014 or the *glkA* mutation, but appeared to reflect the NF status, and

presence of galactose (GAL), galactose plus partially hydrolysed agar (GAL+AGAR), or galactose plus glucose plus partially hydrolysed agar (GAL+GLU+AGAR). See legend to Fig. 1 for abbreviations and additional information.

Fig. 3. S₁ nuclease protection analysis of *dagA* transcription in late exponential phase when cloned in the low copy number plasmid pIJ2014 in *S. coelicolor* A3(2) strains J1508 (*glkA*⁺) and J1376 (*glkA*) (a), M145 and J801 (*SCP1*^{NF}) (b), and *S. lividans* 66 strain TK24 (c). See legends to Figs 1 and 2 for abbreviations and additional information.

presumably resulted from the deletion that gave rise to the *dagA1* mutant allele.

Streptomyces lividans 66 is closely related to *S. coelicolor* A3(2), but does not make agarase. When pIJ2014 was introduced into *S. lividans* 66 strain TK24 by transformation, *dagA* transcription was constitutive, but effectively repressed by glucose (Fig. 3c).

All of these results are consistent with the hypothesis that transcription of *dagA* in *S. coelicolor* A3(2) is negatively regulated by the presence of a specific repressor protein whose activity can be inhibited by partially hydrolysed agar. The gene for this putative repressor is apparently

partially defective in mutant L3, and absent from NF strains of *S. coelicolor* A3(2) and from *S. lividans* 66.

Attempted isolation of the *dagA* repressor gene

Since many regulatory genes are closely linked to the structural genes they control, DNA flanking *dagA* was isolated and assessed for its ability to restore induction of *dagA* transcription by partially hydrolysed agar. Approximately 20 000 plaques from a λ EMBL4 library containing approximately 20 kb fragments of *S. coelicolor* A3(2) M145 chromosomal DNA were probed with the nick-translated 1.5 kb *Bam*HI–*Eco*RI segment of *dagA* that includes the

Fig. 4. S₁ nuclease protection analysis of *dagA* transcription in cultures of *S. coelicolor* A3(2) strain M145 grown on agar plates containing galactose and harvested at the times indicated. See legend to Fig. 1 for abbreviations and additional information.

promoter region and most of the structural gene (Buttner *et al.*, 1987). Twenty hybridizing plaques were purified and re-probed, and DNA was prepared from six randomly chosen clones among the 12 λ EMBL4 derivatives that hybridized most strongly to the probe. Each clone was digested with *Eco*RI, *Eco*RI+*Bam*HI, *Bam*HI and *Pst*I and subjected to Southern analysis with the same 1.5 kb *Bam*HI–*Eco*RI fragment used in the initial screen, and a nick-translated 3.5 kb *Eco*RI–*Bam*HI fragment that contained the remaining 220 bp of C-terminal *dagA* coding sequence and about 3.3 kb of downstream sequence. *Eco*RI fragments extending approximately 10 kb upstream and 15 kb downstream of the unique *Eco*RI site of *dagA* were identified in different λ EMBL4 clones using the N-terminal and C-terminal *dagA* probes, respectively. These fragments were cloned separately into derivatives of pIJ2014 to yield low copy number plasmids that contained *dagA* and either 9 kb of upstream sequence

(pIJ5800) or 15 kb of downstream sequence (pIJ5803). Each plasmid was introduced into *S. coelicolor* A3(2) strain J801 and *S. lividans* 66 strain TK24 by transformation. *dagA* expression in the transformants was assessed by assaying the agarase activity in the supernatants of cultures grown on galactose in the presence or absence of partially hydrolysed agar, and compared to the levels in control cultures of M145. Expression of *dagA* from pIJ5800 was also assessed in both hosts by S₁ nuclease protection studies using RNA isolated from the same cultures that were used for assaying agarase activity. Constitutive expression was observed in all cases, suggesting that the putative *dagA* repressor was not encoded by the cloned flanking regions.

Developmental regulation of *dagA* transcription

The possibility that the four *dagA* promoters were expressed differentially at different stages of morphological development was assessed by S₁ nuclease protection analysis. Since *S. coelicolor* A3(2) does not sporulate in liquid culture, RNA was isolated at different times from M145 mycelium grown on cellophane discs placed on minimal medium agar plates containing galactose. Transcripts originating at p1, p2 and p4 were present throughout growth, but showed a significant and reproducible increase at the onset of aerial mycelium formation (Fig. 4); the level of transcripts attributable to p3 was too low to make any reliable statement about temporal changes in the use of this promoter. As in liquid-grown cultures, transcription of *dagA* appeared to occur predominantly from p1 and p4.

DISCUSSION

Earlier studies demonstrated that agarase production was subject to glucose repression (Hodgson & Chater, 1981), and preliminary data suggested that expression of *dagA* was induced by partially hydrolysed agar (Bibb *et al.*, 1987). Subsequent work revealed that *dagA* was transcribed from four promoters (Buttner *et al.*, 1987) that were recognized, remarkably, by three or four different forms of RNA polymerase holoenzyme (Buttner *et al.*, 1988; Brown *et al.*, 1992). Assuming that the levels of the RNA-protected fragments represent rates of transcription initiation rather than changes in mRNA stability, then we have shown in this paper that in *S. coelicolor* A3(2) strain M145 transcription from all four promoters, which occurs within a region of 187 bp, is induced by partially hydrolysed agar and strongly repressed by glucose.

L3 and L4 produced considerably more agarase on plates than L2 and their progenitor M145. This difference was also evident at the transcriptional level, where the autoradiographs shown in Fig. 2a and b required approximately fivefold longer exposures than those shown in Fig. 2c and d to yield similar band intensities. It appears that expression of *dagA* in the wild-type strain, and in *glkA* mutants derived from it (e.g. L2), cannot be fully induced by partially hydrolysed agar in the presence of the putative *dagA* repressor, and that full induction may be observed only after elimination of the repressor. Inspection of the

profile of RNA-protected fragments obtained for L3 (Fig. 2c) suggests that transcription from p1, p2 and p3 had become constitutive (compare Fig. 2a and c) and, unlike that from p4, no longer inducible by agar degradation products. This raises the interesting possibility that the *dagA* repressor interacts differentially with the p1–p3 and p4 promoters.

Several attempts have been made to define *cis*-acting sequences involved in regulating streptomycete genes that are subject to glucose repression. Mattern *et al.* (1993) identified a complex operator for the galactose-inducible, glucose-repressible *galp1* promoter of *S. lividans* 66 (Fornwald *et al.*, 1987; Ingram *et al.*, 1989). Mutations in a series of hexamers and direct repeats that overlap the RNA polymerase binding site of *galp1* led to inducer-independent expression, although the role of these sequence motifs in glucose repression was not clear. Delić *et al.* (1992) created mutations in a pair of 12 bp direct repeat sequences that again overlapped the RNA polymerase binding site of the chitin-inducible, glucose-repressible promoter of one of the chitinase genes (*chi63*) of *Streptomyces plicatus*. The mutations not only led to chitin-independent expression, but also relieved glucose repression, leading the authors to suggest that the direct repeats constitute an operator for a repressor that is inactivated by chitin, and that glucose repression acts by inducer exclusion (which might also be responsible for glucose repression of *galp1*). Recently, Virolle & Gagnat (1994) identified a region of dyad symmetry located between +9 and +24 with respect to the transcriptional start site of the α -amylase gene (*aml*) of *Streptomyces limosus* which appears to be necessary for both maltotriose induction and glucose repression; deletion of a 29 bp fragment containing the inverted repeat sequence led to inducer-independent expression that was no longer subject to glucose repression. Inspection of the *dagA* promoter region failed to reveal motifs that were strikingly similar to any of the above, although short and different inverted repeats of 5 bp occur just downstream of both the *dagAp4* and *dagAp3* transcriptional start sites; their possible role in the regulation of *dagA* remains to be determined. The identification and characterization of *cis*-acting sequences within the *dagA* promoter region that are required for both aspects of regulation will presumably help to resolve how the four closely located promoters are coordinately regulated. Alternatively, all four promoters may be located in a chromosomal domain that is subject to regulation by DNA looping or supercoiling (Bates & Maxwell, 1993, and references therein).

Hodgson (1982) had originally shown that glucose repression of agarase activity, and of the expression of several other glucose repressible genes, occurred in a glucose kinase-dependent manner. In principle, glucose kinase might mediate its effect on all of these systems by inducer exclusion; the repression of arabinose and glycerol transport, and the inhibition of galactose uptake, are all relieved in *glkA* mutants (Hodgson, 1982). However, glucose repression of *dagA* is maintained under conditions of constitutive, i.e. inducer-independent, expression (e.g. Fig. 2d, 3a and 3c), suggesting that inducer exclusion does

not play a central role in glucose repression of *dagA* transcription. Similar conclusions can be drawn for the α -amylase gene (*aml*) of *S. limosus*. When cloned in *S. coelicolor* A3(2) at low copy number, transcription of *aml* was induced by maltotriose, but at high copy number transcription became constitutive, presumably through titration of a repressor protein (Virolle & Bibb, 1988); however, glucose repression was retained regardless of whether transcription was inducible or constitutive. The lack of an apparent role for inducer exclusion in glucose repression of *dagA*, and perhaps *aml*, and the possibility that it is responsible for glucose repression of *chi63* and *galp1*, might reflect the operation of different mechanisms of carbon catabolite repression. It is thus pertinent to note that glucose represses the expression of both *chi63* (C. Ingram & J. Westpheling, personal communication) and the xylose utilization operon of *Streptomyces rubiginosus* (Wong *et al.*, 1991) in both *glkA*⁺ and *glkA*[−] derivatives of *S. coelicolor* A3(2).

dagA is transcribed from four different promoters that show little sequence similarity, by at least three, and probably four, different RNA polymerase holoenzymes, each containing a different σ factor. The reasons for such a complex transcriptional arrangement are not understood, but it is tempting to speculate that it might reflect the changing availability of different holoenzyme forms during growth and differentiation. The experiments conducted here failed to provide evidence in favour of this notion, although the physiological heterogeneity that almost certainly exists as a consequence of mycelial growth, and the lack of synchrony during development of surface-grown cultures, might make such data difficult to obtain. Alternatively, the failure to detect differences in the use of the four promoters might reflect the limited circumstances experienced by the strain in the laboratory, and our inability to reproduce conditions encountered in the natural environment that might trigger differential expression.

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