A Chromosomal Locus Controlling Extracellular Agarase Production by *Streptomyces coelicolor* A3(2), and its Inactivation by Chromosomal Integration of Plasmid SCP1

By D. A. HODGSON† AND K. F. CHATER*

John Innes Institute, Colney Lane, Norwich NR4 7UH

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The properties of various mutants showed that, in the presence of an inorganic nitrogen source, agar utilization by *Streptomyces coelicolor* requires the action of at least three groups of enzymes: a diffusible or cell-free extracellular agarase, some further degradative step(s), and the enzymes of galactose metabolism. Two unselected mutations (*dag*), both leading to loss of diffusible agarase and an undefined second agar utilization function, were detected among a small number of *S. coelicolor* derivatives from a culture collection, and mapped to the 9 o'clock 'silent' region of the genetic map. One of these mutations (*dagA1*) had apparently resulted, directly or indirectly, from the insertion of the plasmid SCP1 in this region during formation of the NF fertility type. The other (*dag-2*) was 100% linked to *dagA1* and the integrated SCP1, but only 98% linked to the site of insertion of SCP1 in an 'NF-like' but Dag⁺ strain. SCP1–chromosome interactions in other regions of the genetic map had no effect on the agar-solubilizing phenotype, nor had three other unstable genetic phenomena in *S. coelicolor*.

INTRODUCTION

Agar is a polysaccharide of D- and L-galactose-related compounds (Yaphe & Duckworth, 1972) capable, at a concentration of about 1%, of forming the rather rigid gels familiar to microbiologists. The conversion of agar in gel form to a mixture of oligosaccharides that can be utilized by microbial cells requires the production of extracellular enzymes, which may be diffusible (e.g. *Pseudomonas atlantica*; Groleau & Yaphe, 1977) or trapped in secreted slime (e.g. *Cytophaga flevensis*; see van der Meulen & Harden, 1976). Most of the known agarases cleave the 1,4- β -linkage of agarose to give oligosaccharide multimers of neoagarobiose, which may then be broken down at the cell membrane or transported into the cell and broken down intracellularly, and finally modified to give products metabolized by the enzymes of galactose metabolism. There is some evidence that *C. flevensis* agarase may be induced by agar and some of its breakdown products and repressed by glucose (van der Meulen & Harden, 1975).

Our interest in agar utilization was stimulated by our genetic investigations of carbon metabolism in the genetically well-studied strain A3(2) of *Streptomyces coelicolor*: such studies have been hindered by the organism's ability to use agar as sole carbon source (Hopwood *et al.*, 1973). In fact, early reports of the progenitor of this strain, called NB 3443, concerned its agar-degrading ability and its growth on agar as sole carbon source (Stanier, 1942).

The investigations reported here have revealed an apparent interaction between the plasmid SCP1 and a gene (or genes) concerned in agar degradation, so it is relevant to describe

† Present address: Department of Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, New York 10461, U.S.A.

certain aspects of SCP1 genetics. The wild-type strain carries this highly transmissible plasmid in an autonomous state, in which it also acts as an inefficient sex factor (Vivian, 1971). Rarely, the plasmid recombines with the chromosome to give strains which donate, at high frequency, markers near the interaction site to SCP1⁻ strains. Some of these donor strains contain SCP1-prime plasmids (Hopwood & Wright, 1973, 1976*a*), and in others SCP1 is integrated into the chromosome at one of a number of possible sites (Vivian & Hopwood, 1970, 1973; Hopwood & Wright, 1976*b*). The latter class may be formed predominantly through interactions between SCP1-prime plasmids and the chromosome (Hopwood & Wright, 1976*a*). Particular reference will be made to two kinds of strains with an integrated SCP1 plasmid: NF, which occurred, spontaneously, early in genetic investigations of *S. coelicolor* (Hopwood *et al.*, 1969); and the 'NF-like' strain A634, obtained as a stable derivative of an unstable donor strain which was itself probably an SCP1-prime strain (Vivian & Hopwood, 1973). In both kinds of strains SCP1 is integrated in the 9 o'clock 'silent' region of the chromosome, and this causes high frequency donation to SCP1⁻ strains of markers on both sides of the integration site (i.e. bidirectional donation).

In this paper we report genetic and physiological aspects of agar catabolism, and the deduction that chromosomal integration of the plasmid SCP1 in one case inactivated a gene involved in agar catabolism.

METHODS

Organisms. All strains were recombinational and mutational derivatives of *Streptomyces coelicolor* A3(2) (Table 1).

Media and general methods of culture, mutagenesis and genetic analysis. These were as described by Hopwood (1967) and Hopwood *et al.* (1973). Variations were sometimes introduced into the minimal agar medium (MM) as follows: NMM, asparagine omitted, 0.1% (w/v) (NH₄)₂SO₄ added; NO₃MM, asparagine omitted, 0.1% (w/v) KNO₃ added. Carbon sources additional to agar, when present, were at a final concentration of 1% (w/v).

Testing for integrated plasmid SCP1. Analysis of recombinants for NF (or 'NF-like') donor properties was done by replica plating of patches to a lawn of strain 2369 on complete medium, incubating for 4 d, and replicating to supplemented MM selecting for recombinants. Confluent growth of recombinants on the selective medium indicated the presence of the donor property. The use of the rifampicin resistance marker of strain 2369 meant that even prototrophic, streptomycin-resistant strains could be subjected to this test.

Gran's test for agar decomposition. The plate was flooded with 0.05 M-iodine in 0.12 M-KI. Agar decomposition was seen as a clearing (pale yellow/brown) in the dark brown background, which was emphasized if the plate was kept at 4 °C for several hours. In an experiment to test for the ability of agarase to pass through dialysis membrane, a 4 × 5 cm piece of membrane (Visking, 32/32, prewashed by boiling in phosphate buffer) was dipped into molten MM lacking glucose and laid on the surface of MM lacking glucose. A drop of spore suspension (about 10⁷ spores) was placed on the membrane. After 13 d the plate was subjected to Gran's test both before and after removal of the dialysis membrane.

Washing of agar. Lab M agar powder (MC2; London Analytical and Bacteriological Media, London) was suspended in an equal volume of 5 M-KCl and poured into a column. After draining, the column was washed with distilled water until the effluent reached a steady conductivity (15 mS, i.e. three times higher than the input distilled water). The agar was then lyophilized and sterilized either by soaking in chloroform, which was allowed to evaporate before use of the agar in plates, or by autoclaving the medium after addition of the agar.

Tests for other extracellular hydrolytic enzymes. Substrates for extracellular enzymes were incorporated in MM and NMM with and without glucose, as follows: starch (1 and 0.1%, w/v), gelatin (B.D.H.; 1%, w/v), pectin (0.5%, w/v), skimmed milk (10%, w/v) and tributyrin (Sigma; 1%, w/v). Strains to be tested were incubated on these media for at least 4 d before scoring. Starch degradation was tested by staining with iodine; gelatin degradation by acidic (5 M-HCl) HgCl₂ precipitation of gelatin (also by sinking of colonies into 15% gelatin gels); pectin degradation by cetrimide precipitation of pectin; and milk and tributyrin degradation by visual inspection for zones of clearing.

RESULTS

Identification and some regulatory aspects of diffusible extracellular agarase

Strain J800 (SCP1⁻) grew, albeit to a reduced extent, on MM in the absence of glucose. Since the nitrogen source in MM is asparagine (a potential source of carbon), we replaced it

Table 1. *Streptomyces coelicolor* strains used

Strain no.	Genotype	SCP1 status†	SCP2 status‡
4	<i>hisA1</i>	NF	*
12	<i>pheA1</i>	+	+
104	<i>hisA1 uraA1 strA1</i>	+	+
283	<i>hisD3 cysB6 strA1</i>	+	+
949	<i>proA1 argA1 cysA15 uraA1 nicA1 tps30</i>	NF	*
1098	<i>pheA1</i>	—	+
1147	parental wild-type	+	+
1190	<i>hisA1 uraA1 strA1</i>	—	+
1511	<i>proA1 argA1 cysA15 uraA1 nicA1 Aga⁻</i>	NF	*
1514	<i>proA1 argA1 cysA15 uraA1 nicA1 agaA7</i>	NF	*
1873	<i>pheA1</i>	SCP1' <i>cysB</i> ⁺	+
1956	<i>hisD3 cysB6 strA1</i>	—	+
1984	<i>hisD3 cysB6 strA1</i>	SCP1' <i>cysB</i> ⁺	+
2106	<i>pheA1</i>	<i>cysD</i> UD	+
2278	<i>proA1 argA1 cysD18</i>	+	+
2293	<i>hisD3 cysB6 strA1</i>	<i>cysB</i> BD	+
2369	<i>hisA1 uraA1 strA1 rif</i>	—	+
2697	<i>proA1 argA1</i>	NF	*
A303	<i>hisA1 uraA1 strA1</i>	NF	+
A317	<i>hisA1 uraA1 strA1</i>	NF	*
A321	<i>uraA1</i>	NF	*
A479	<i>adeCv10 pheA1 strA1</i>	+	+
A608	<i>pheA1</i>	<i>pabA</i> UD	+
A634	<i>proA1 argA1 cysA15 strA1</i>	'NF-like'	?
B52	<i>hisA1 uraA1 strA1 cre-1</i>	—	*
B261	<i>argA1 guaA1 cre-1</i>	—	*
B311	<i>pabA1 mthB2 hisD3 guaA1 cysD18 strA1</i>	—	*
J650	<i>mthB2 cysD18 agaA7</i>	NF	*
J800	+	—	+
J801	+	NF	*
J802	<i>agaA7</i>	NF	*
J825	<i>hisD3 cysB6 strA1</i>	+	+

† SCP1 status symbols: NF, plasmid SCP1 integrated into the chromosome in the 'Normal Fertility' mode, *sensu* Hopwood *et al.* (1969); SCP1' *cysB*⁺, autonomous SCP1 containing the normally chromosomal *cysB*⁺ gene; *cysD* UD, plasmid SCP1 integrated, giving apparently unidirectional chromosome transfer with *cysD* as an early marker (Hopwood & Wright, 1976*b*); *cysB* BD, plasmid SCP1 integrated near the *cysB* gene, giving bidirectional chromosome transfer (Hopwood & Wright, 1976*a*); *pabA* UD, plasmid SCP1 integrated, giving unidirectional chromosome transfer with *pabA* as an early marker (Vivian & Hopwood, 1973); 'NF-like', independently obtained SCP1 integration giving properties very similar to NF (Vivian & Hopwood, 1973).

‡ SCP2 status symbols: SCP2*, variant of SCP2 with enhanced sex factor activity (Bibb *et al.*, 1977). All the NF strains except A303 are presumed to contain SCP2* because this property was common to all of a large number of NF strains tested by M. J. Bibb (personal communication). Strain A303 is one of a class of NF recombinants obtained in NF SCP2* × SCP1⁻ SCP2⁺ crosses which apparently have inherited SCP2⁺ rather than SCP2* (M. J. Bibb, personal communication).

by ammonium (giving NMM). Growth still took place. This result suggested that strain J800 (SCP1⁻) could utilize agar as sole carbon source, although it did not eliminate the possibility that an impurity or degradation product present in the agar was being utilized. (Indeed, chloroform-sterilized washed agar did not serve as a carbon source for growth, though autoclaved washed agar did. This was probably due to a requirement for agar degradation products for induction of agarase.)

Growth of strain J800 (SCP1⁻) on NMM or MM in the absence of glucose was accompanied by noticeable sinking of the colonies into the medium, suggestive of extracellular agar degradation. This was more clearly visualized by applying Gran's iodine test, which revealed a wide zone of clearing round the colonies. Agarase zones were not manifested through dialysis membrane (see Methods). This observation was consistent with

the expectation that enzymic agar degradation was involved. Possession of this property was designated the Dag (for *diffusible agarase*) phenotype.

A negative Gran's test was obtained when NMM (but not MM) was supplemented with glucose. This suggested that a form of glucose repression might influence diffusible agarase production, and that this glucose repression system interacted with nitrogen metabolism. When the effects of a number of other carbon sources on agarase production were tested, repression (when it occurred at all) was again seen on NMM but not on MM. The repressing compounds were cellobiose, mannose and rhamnose, while no repression was observed with fructose, galactose, gluconate, glycerol, maltose, mannitol or xylose. Arabinose showed apparent repression on a few occasions in repeated tests.

Strains of NF fertility type are Dag⁻

Strain J801 (NF) grew more poorly than J800 (SCP1⁻) on MM containing either asparagine or arginine (added at the same concentration, w/v, as asparagine) as nitrogen source and not at all on NMM or NO₃MM with agar as sole carbon source. (This was true also when the medium was buffered with 20 mM-phosphate buffer, pH 7.1, eliminating trivial pH effects as a cause of these different responses.) Ammonium did not actively repress or inhibit agar utilization, since NMM containing asparagine but not glucose supported growth of J801 (NF).

Colonies of J801 (NF) did not sink into the agar, and they gave a negative Gran's test, i.e. they were Dag⁻. Examination of a number of strains varying in chromosomal markers and SCP1 plasmid status (SCP1⁻ strains 1190, 1098, B261, B52, J800; SCP1⁺ strains 12, 104, 1147; NF strains J650, J801, J802, A303, A321) showed that all the NF strains, but none of the SCP1⁺ or SCP1⁻ strains, in this particular test were Dag⁻. Since all NF strains are descended from one of the progeny of a cross in which the SCP1 integration event giving NF fertility apparently first occurred (Vivian & Hopwood, 1970), the oldest available NF strain (strain 4) was tested. It, too, was Dag⁻, suggesting a causal relationship between the SCP1 integration event and the Dag⁻ phenotype. The mutation causing the Dag⁻ phenotype of NF strains was designated *dagA1*.

Complete linkage of NF and the Dag⁻ phenotype

In crosses between NF and SCP1⁻ strains essentially all the progeny are NF, and the further away a chromosomal marker is from the 9 o'clock region (in which SCP1 is integrated in its NF mode), the lower its frequency of inheritance among the progeny (Hopwood *et al.*, 1969). When NF strain 949 (*dagA1*) was crossed with strain J800 (SCP1⁻, *dag*⁺), all the NF recombinants obtained were Dag⁻, suggesting that *dagA1* mapped close to the 9 o'clock region. This was confirmed in a cross between strains A317 (NF, *dagA1*) and 2278 (SCP1⁺, *dag*⁺) (Fig. 1). In such NF × SCP1⁺ crosses, obligate inheritance of NF does not occur, and the progeny segregate into the alternative NF and SCP1⁺ states, with the difference mapping at 9 o'clock (Vivian & Hopwood, 1970). As predicted, complete co-segregation of NF and *dagA1* was observed and in several further such crosses no recombination between NF and *dagA1* has ever been seen.

Chromosomal integration of SCP1 does not inevitably cause the Dag⁻ phenotype

The Dag⁻ phenotype might have resulted from direct inactivation of a chromosomal gene into or near which SCP1 had integrated, or from some general consequence of interaction between SCP1 and the chromosome. Four strains showing interactions between SCP1 and different regions of the chromosome were subjected to Gran's test. These were: (1) strain A608, a stable unidirectional *pabA*⁺ donor (Vivian & Hopwood, 1973); (2) strain A634, an 'NF-like' strain that resembled NF strains in its fertility properties, though of independent origin (Vivian & Hopwood, 1973); (3) strain 2106, a stable unidirectional *cysD*⁺ donor (Hopwood & Wright, 1976*b*); and (4) strain 2293, a bidirectional *cysB*⁺ donor (Hopwood &

Fig. 1. Crosses between strain A317 (*dagA1*, NF) (outer circle) and strain 2278 (*dag*⁺, SCP1⁺) (inner circle) to investigate segregation of *dagA1*: (a) selection (▲) for *proA*⁺ *uraA*⁺ recombinants; (b) selection (▲) for *str*^R *hisA*⁺ recombinants. Numbers indicate allele frequencies. All NF recombinants were *dagA1* and all SCP1⁺ strains were *dag*⁺ for each selection.

Fig. 2. The Dag status of the progenitors of strain 2293 (pedigree provided by D. A. Hopwood).

Wright, 1976a). All but strain 2293 proved to be Dag⁺, indicating that chromosomal integration of SCP1 did not inevitably result in inactivation of the gene(s) required for the Dag⁺ phenotype.

The pedigree of strain 2293, which was Dag⁻, is shown in Fig. 2. Its progenitors were subjected to Gran's test, and strains 1956 and 1984 (but not 283 or 1873) were found to be Dag⁻. This indicated that a mutation to Dag⁻ had occurred at or around the time of u.v.-induced curing of SCP1 from strain 283, and thus that the Dag⁻ phenotype of strain 2293 was not connected with its integrated SCP1 plasmid. This mutation was designated *dag-2*.

Fig. 3. Mapping of *dag-2*. Strain 1956 (*dag-2*, SCP1-SCP2⁺) (outer circle) was crossed with strain B311 (*dag*⁺, SCP1-SCP2^{*}) (inner circle): selection (▲) for *cysB*⁺ *mth*⁺ recombinants. Numbers indicate allele frequencies.

Mapping of the *dag-2* mutation

Strain 1956 (*dag-2*, SCP1-SCP2⁺) was crossed with the multiply marked *dag*⁺SCP1-SCP2^{*} strain B311 (Fig. 3). Crosses between SCP2⁺ and SCP2^{*} strains (in the absence of SCP1) are usually symmetrical and non-polarized and give a rather high fertility level (Bibb *et al.*, 1977; Bibb, 1978). They are therefore well suited to the preliminary mapping of new mutations. In this particular cross, the frequency of the *dag-2* allele among the recombinants suggested that it was located either between *cysB* and *guaA* (in one of the arcs separating the selected markers), or between *cysB* and *pabA* (in the other arc). Segregation of *dag-2* and *guaA* was not significantly different from independence [$\chi^2_1 = 1.1(5)$; $P = 0.3$], whereas segregation of *dag-2* and *pabA* departed significantly from independence [$\chi^2_1 = 16$; $P \leq 0.001$]: this placed *dag-2* in the *cysB* to *pabA* arc.

This location for *dag-2* raised the possibility that it might be closely linked to the *dagA* locus and therefore to the site of SCP1 integration in NF strains: the cross shown in Fig. 4 was used to examine this possibility. The aim was to seek *dag*⁺ recombinants in this *dagA1* × *dag-2* cross. Two features of the cross were particularly important: the use of an SCP1⁺ strain carrying *dag-2*, so that the inheritance of NF (and therefore of *dagA1*) by all the progeny (an obligatory feature of crosses between NF and SCP1⁻ strains) could be avoided; and the possibility of imposing two alternative patterns of selection, so that in at least one of the selections the generation of *dag*⁺ recombinants would not require multiple crossing over. Among about 100 recombinants analysed in detail from each selection none was *dag*⁺. The resolution of this analysis was then increased by classifying larger numbers of recombinant colonies only for their *dag* status: recombinants were replicated to MM without glucose and, after 7 d incubation, subjected to Gran's test. No *dag*⁺ recombinants were obtained among about 1000 recombinants for the *proA*⁺*cysB*⁺ selection and about 2000 for the *hisD*⁺*strA1* selection. Thus *dagA1* and *dag-2* were very closely linked and probably allelic.

Mapping of the site of SCP1 integration in A634

Strain A634, previously apparently indistinguishable from NF strains though of independent origin (Vivian & Hopwood, 1973), was shown (above) to differ from NF strains in being *Dag*⁺. By crossing A634 with a *dag-2*SCP1⁻ strain we could seek recombinants between the SCP1 integration site of A634 and *dag-2*, and therefore find out whether this

Fig. 4. Attempt to produce *dag*⁺ recombinants by crossing strain 2697 (*dagA1*, NF) (outer circle) and strain J825 (*dag-2*, SCP1⁺) (inner circle): (a) selection (▲) for *pro*⁺ *cys*⁺ recombinants; (b) selection (▲) for *str*^R *his*⁺ recombinants. Numbers indicate allele frequencies. No *dag*⁺ recombinants were found in either selection among the recombinants subjected to full analysis, nor among about 1000 (otherwise unanalysed) *pro*⁺ *cys*⁺ recombinants or among about 2000 (otherwise unanalysed) *str*^R *his*⁺ recombinants.

Fig. 5. Crossing over between *dag-2* and the SCP1 integration site in the 'NF-like' strain A634. Strain A634 (*dag*⁺, 'NF-like') (outer circle) was crossed with strain 1956 (*dag-2*, SCP1⁻) (inner circle) (an ultrafertility cross; Vivian & Hopwood, 1973): (a) selection (▲) for *cysA*⁺ recombinants (by growth on thiosulphate on which *cysB* mutants can grow, but *cysA* mutants cannot; Hopwood, 1967); (b) selection (▲) for *cysA*⁺ *cysB*⁺ recombinants. Numbers indicate allele frequencies. In both analyses, all the recombinants contained integrated SCP1.

site mapped separately from the integration site in NF strains. In a preliminary non-selective analysis of the ultrafertility cross between A634 and the *dag-2* SCP1⁻ strain 1956, all 97 recombinants were *dag*⁺ and had the A634 fertility type, confirming that the SCP1

integration site of A634 was closely linked to *dag-2* (especially in view of the steep allele gradient observed). The resolution of the analysis was increased by imposing two nutritional selections, shown in Fig. 5, in addition to the unavoidable 'natural' selection of recombinants inheriting the integrated SCP1. Rare (4/137) recombinants that were *dag-2* could then be detected among recombinants arising by crossing over between SCP1 and *cysB* (Fig. 5a), whereas none were found when crossing over in this interval was selected against (Fig. 5b). This result suggested that SCP1 in strain A634 was integrated into the chromosome at a site closer to *cysA* than that occupied by SCP1 in NF strains (or alternatively that the two plasmid integration sites were congruent but a deletion in NF strains removed DNA separating the plasmid integration site from the *dagA* locus).

Possible instability of the Dag phenotype and its independence of various unstable genetic phenomena in S. coelicolor

Since two independent, spontaneous, unselected occurrences of mutations to Dag⁻ had been detected in a very limited search of the John Innes Institute *Streptomyces* culture collection, it could be considered to be a genetically unstable character. Attempts were therefore made to isolate more Dag⁻ mutants. After u.v. irradiation of spores, about 7200 colonies of strain 104, about 3000 of strain 283, and about 2800 of strain 1190, were all Dag⁺. Revertants of *dagA1* in J801 to Dag⁺ were also sought, by selecting for the ability to grow on NMM without glucose. None were obtained either spontaneously (out of about 4.8×10^6 spores plated) or after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (out of about 1.07×10^4 spores plated).

The possibility that Dag status might be connected with genetic instability of other characters was also tested. These characters were: the chloramphenicol resistance-sensitivity-resistance oscillation (Freeman *et al.*, 1977); loss of resistance to phage ϕ C31, which occurs at high frequency (about 1%) after u.v. irradiation (Lomovskaya *et al.*, 1980); and changes in status with respect to plasmid SCP2, which may spontaneously be lost (to give SCP2⁻) at a frequency of about 1%, or give rise to the variant form SCP2* (Bibb *et al.*, 1977). None of these affected the Dag phenotype.

Other mutations affecting agar utilization

Hopwood *et al.* (1973) described the isolation and preliminary mapping of mutants (*aga*) unable to utilize agar as sole carbon source on MM (the Aga⁻ phenotype). These mutants were all isolated in an NF, and therefore (as we now know) *dagA1*, genetic background. We may consider three possible roles for the *aga* gene product(s): (a) involvement in degradation of agar to galactose-related monomers; (b) involvement in galactose metabolism; or (c) scavenging of amino acids as sole carbon sources, and thus complete lack of involvement in agar metabolism. At least one of the mutants (1514, containing the *agaA7* mutation at a map location between *cysA* and *adeC*, i.e. not closely linked to the *dagA* locus) was clearly defective in role (a), because it was possible by recombination to construct *dagA*⁺*agaA7* strains which were unable to utilize agar as sole carbon source on MM. [The cross used to obtain such recombinants was between the SCP1⁺ *dag*⁺ *aga*⁺ strain A479 (*adeCv10 pheA1 strA1*) and the NF *dagA1 agaA7* strain 1514 (*argA1 proA1 cysA15 nicA1 uraA1*). With selection for *uraA*⁺ *nicA*⁺ *adeC*⁺ *strA1* recombinants, 10 of 145 colonies scored gave a positive Gran's test on MM plus glucose but were unable to grow on agar as sole carbon source.] Another Aga⁻ mutant, 1511, proved to owe its Aga⁻ phenotype to a defect in role (b) – it was unable to utilize galactose (and was indeed galactose-sensitive).

Cross-feeding tests

Strains that were *dagA1 agaA*⁺ or *dag-2 agaA*⁺ were not stimulated to grow better on NMM lacking glucose by inoculation close to a *dag*⁺*aga*⁺ strain, even when within an

Table 2. Summary of phenotypes of mutants affected in agar utilization

Relevant genotype	MM			NMM		
	Growth on unwashed agar as sole carbon source	Dag phenotype		Growth on unwashed agar as sole carbon source	Dag phenotype	
		Glucose absent	Glucose present		Glucose absent	Glucose present
<i>dag⁺ aga⁺</i>	++	+	+	++	+	—
<i>dagA1</i> (NF) <i>aga⁺</i>	+	—	—	—	—	—
<i>dag⁺ agaA7</i>	—	+	+	—	+	—
<i>dagA1</i> (NF) <i>agaA7</i>	—	—	—	—	—	—

agarase 'field', nor was cross-feeding of strain J802 (*agaA7 dagA1*) by strain J800 (*aga⁺ dag⁺*) detected.

The dag mutants are not pleiotropically defective in extracellular enzymes

Strains J800 (*aga⁺ dag⁺*), J801 (*aga⁺ dagA1*) and J802 (*agaA7 dagA1*) were all indistinguishable from the parental strain (1147) in their production of extracellular enzymes involved in digestion of starch, gelatin, pectin, skimmed milk or tributyrin.

DISCUSSION

Three types of *S. coelicolor* mutation impairing agar utilization have been identified: one (present in strain 1511) prevented galactose utilization and caused galactose sensitivity; another (*agaA7*) prevented growth on agar as sole carbon source apparently without affecting any other pathway(s), yet did not inactivate agarase (Hopwood *et al.*, 1973, and this report); and a third (*dag*), described in this report, inactivated extracellular agarase (without inactivating other extracellular enzymes).

The ability of *S. coelicolor* to use agar requires not only a complete galactose utilization system and the product(s) of the *dag* gene(s) mapping in the 9 o'clock regions, but also the product of the *agaA⁺* gene (Table 2). This implies that *agaA* has a specific agar utilization function. The growth of *agaA⁺* but not of *dagA⁺ agaA7* strains only on unwashed agar, or on washed agar that had been autoclaved, suggests that the *agaA* gene product may allow scavenging of agar degradation products accumulated during storage and autoclaving. These were not the same products as occur on agar digestion by agarase, however, because *dag⁺* strains could not cross-feed *dag* strains. The poor growth of *dag⁺ aga⁺* strains on chloroform-sterilized washed agar could be explained by the absence of inducers of the *dagA* gene, which may have been washed out of the agar. The suggestion that *dagA agaA⁺* strains scavenge for agar degradation products may explain the effect of nitrogen source on their growth. Asparagine and arginine may act as carbon sources as well as nitrogen sources and so could supplement the carbon supplied by scavenging for agar degradation products (although neither asparagine nor arginine could act as sole carbon source).

Of the two *dag* mutations described, one (*dagA1*) appears to have resulted from the inactivation of a gene required for diffusible agarase production, by insertion of SCP1 into the 9 o'clock region of the chromosome. The other (*dag-2*) may have resulted from a spontaneous or u.v.-induced mutation of the same gene, though it is not excluded that the mutational event was somehow related to the loss of SCP1 which was detected at about the same time (in which case our attempt to isolate more *dag* mutants might profitably have been concentrated only on those survivors of u.v.-irradiation which had lost SCP1). Since neither *dagA1* nor *dag-2* strains could be cross-fed by a *dag⁺* strain, they must be defective in some function(s) additional to the agarase, since the products of agarase action should have been

available to any cell in the vicinity. Thus, they might also have lost a transport function or one of the intracellular enzymes for degrading the products of agarase action (assuming the system to be similar to that in other bacteria). This may be explained in several ways: (1) the *dag* mutants may contain deletions of the agarase structural genes and the secondary function gene(s); (2) a mutation of either the agarase structural gene or the secondary function gene(s) caused polar effects on expression of nearby genes; (3) a regulatory or processing gene affecting several agar-digesting enzymes had been inactivated; or (4) loss of a cofactor or subunit common to the agarase and to the other function(s) was the primary cause of the phenotype.

The simultaneous loss of both functions in both of the chance events that gave rise to the *Dag*⁻ phenotype, the unusual location of *dag* in the otherwise genetically 'silent' 9 o'clock region of the chromosome, and the insertion of SCP1 close to or within *dag*, all combine to suggest that there may be something special about the *dag* region. One obvious possibility is that DNA sequence homology between SCP1 and the chromosome may be present in this region, and be involved both in SCP1 integration and in generating deletions of nearby genes. By analogy with interactions between the F plasmid and the *Escherichia coli* chromosome, sequences resembling IS elements might be implicated (Hu *et al.*, 1975).

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