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The Agarase Gene (*dagA*) of *Streptomyces coelicolor* A3(2): Affinity Purification and Characterization of the Cloned Gene Product

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The coding and regulatory sequences of the agarase gene of *Streptomyces coelicolor* A3(2) were cloned in *Streptomyces lividans* 66 on the plasmid vector pIJ61, resulting in a several hundred-fold increase in the production of the secreted protein. Subcloning experiments localized the sequences required for agarase production and for the mediation of carbon catabolite repression to a segment of about 1·2 kb. A simple protein purification procedure that uses affinity binding of agarase to agarose beads was developed. Preliminary characterization of the enzyme, together with the results of *in vitro* transcription–translation studies, suggest that the intracellular form of agarase (about 34 kDa) possesses a signal sequence that is cleaved upon secretion across the cell membrane to produce an extracellular protein of about 29 kDa.

INTRODUCTION

Streptomyces coelicolor A3(2) is one of only a few bacterial species that can utilize agar as sole carbon source (Stanier, 1942; Buchanan & Gibbons, 1974). Agar is a highly heterogeneous polysaccharide, of which the simplest form, neutral agarose, is an alternating polymer of D-galactose and 3,6-anhydro-L-galactose linked by alternating $\beta 1 \rightarrow 4$ and $\alpha 1 \rightarrow 3$ bonds (Yaphe & Duckworth, 1972). In agar many of these sugar residues are replaced by substituted derivatives and neutral agarose probably represents only a minor component of the natural polymer. Although the biochemistry of agar utilization has not been studied in *S. coelicolor*, it is apparent from genetic analysis of this organism (Hodgson & Chater, 1981) and from studies of agar utilization in other bacteria, particularly *Pseudomonas atlantica* (Morrice *et al.*, 1983) and *Cytophaga flevensis* (van der Meulen & Harder, 1976), that a number of different enzymes are required. The first stage of agar degradation generally involves an extracellular diffusible agarase (the Dag^+ phenotype) which is responsible for reducing the macromolecular complex to a series of smaller oligosaccharide multimers of neoagarobiose (3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose) which then form the substrates for further enzymes involved in agar utilization. These enzymes ultimately yield the constituent monomers 3,6-anhydro-L-galactose and D-galactose. While the fate of 3,6-anhydro-L-galactose is unknown, the inability of *gal* mutants of *S. coelicolor* to use agar as the sole carbon source implies that all the utilized carbon derived from agar is incorporated via D-galactose (Hodgson & Chater, 1981).

Earlier genetic studies in *S. coelicolor* had identified several mutants, in addition to those involved in galactose metabolism, that were deficient in their ability to utilize agar for growth.

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Abbreviation: CIAP, calf intestine alkaline phosphatase.

These included mutations in the *aga* locus, which appear to be responsible for further processing or scavenging of agar degradation products (Hopwood *et al.*, 1973; Hodgson & Chater, 1981), as well as two independent Dag$^-$ mutations that mapped some distance from *aga* in the unusual silent region of the chromosome between *uraA* and *pabA* (Hodgson & Chater, 1981); both failed to segregate from the integrated form of the SCP1 plasmid found in *S. coelicolor* NF strains. One, *dagA1*, has since been shown to have undergone a deletion of the entire agarase gene (*dagA*) that presumably occurred during plasmid integration to give the original NF strain (Kendall & Cullum, 1986). The *dagA1* mutants cannot be cross-fed by Dag$^+$ strains (Hodgson & Chater, 1981). This implies that another gene (or genes) essential for growth on agar and whose product is presumably cell-bound, was affected by this deletion event and possibly reflects the existence of a cluster of genes involved in agar utilization in the *dagA* region.

The *dagA* gene of *S. coelicolor* shows some interesting regulatory features. It is subject to carbon catabolite repression (Hodgson & Chater, 1981) by a mechanism that appears to be quite distinct from that observed in enteric bacteria (Adhya & Garges, 1982: Botsford, 1981; Chatterjee & Vining, 1982; Cortes *et al.*, 1986; de Crombrugghe *et al.*, 1984; Hodgson, 1982; Martin & Demain, 1980; Surowitz & Pfister, 1985), and preliminary studies have indicated that *dagA* is also inducible by the products of partial agar hydrolysis (R. Joseph, unpublished results).

The experiments reported here were undertaken to initiate a study of the structure and regulation of the *dagA* gene of *S. coelicolor*, to provide a basis for further studies on carbon catabolite repression in *Streptomyces* and to yield information on the process of protein secretion in members of this genus. We describe the cloning of the *dagA* coding and regulatory sequences and the purification and preliminary characterization of the gene product. The cloning of *dagA* from *S. coelicolor* and some analysis of the gene product have also been reported by Kendall & Cullum (1984).

METHODS

Bacterial strains and plasmids, transformation and cultural methods. Standard media and methods of culture for *S. lividans* and *S. coelicolor* and conditions for transformation of and cloning in *S. lividans* TK24 (Hopwood *et al.*, 1983) were as described by Hopwood *et al.* (1985). M145 is a prototrophic SCP1$^-$ SCP2$^-$ derivative of *S. coelicolor* A3(2) (Hopwood *et al.*, 1985). pIJ61 (Thompson *et al.*, 1982), which has a copy number of 5–6 per chromosome, was used as cloning vector. pMT605 and pMT608 (Kendall & Cullum, 1984) are *dagA*-containing derivatives of the high copy number plasmid pIJ702 (Katz *et al.*, 1983). For agarase purification, cultures were grown for 60–72 h at 30 °C in L-broth (Lennox, 1955) containing, with the exception of M145, 10 μg thiostrepton ml^{-1}. The mycelium was separated from the culture supernatant by suction filtration through Whatman no. 1 filter paper. To assess carbon catabolite repression of agarase production the modified minimal medium (NMM) of Hodgson & Chater (1981) was used. Unless otherwise stated, sugars were added to NMM to give a final concentration of 1% (w/v).

DNA isolation, manipulation and characterization. Plasmid DNA was prepared either by the method of Kieser (1984) or by a modification (S. Y. Chang, personal communication; Hopwood *et al.*, 1987) of the method of Ish-Horowicz & Burke (1981). Total DNA was isolated as described by Hopwood *et al.* (1985). Restriction endonucleases and T4 DNA ligase (Anglian Biotechnology or BRL) and calf intestine alkaline phosphatase (CIAP) (Boehringer–Mannheim) were used according to the manufacturers' recommendations. Agarose gel electrophoresis was carried out as described by Hopwood *et al.* (1985) and the sizes of DNA fragments were estimated using the program of Duggleby (1981) modified for use on an Acorn BBC model B microcomputer (T. H. N. Ellis & M. J. Bibb, unpublished).

Agarase purification. (i) *Enzyme assay.* The agarase assay was based on the production of reducing sugar resulting from the action of the enzyme on agarose (Dygert *et al.*, 1965). Reaction mixtures (1 ml) contained: 0·1% agarose (Sigma); 50 mM-imidazole/HCl pH 6·5; and enzyme. Reaction mixtures were incubated for 15 min at 37 °C, after which 1 ml copper sulphate solution (4%, w/v, Na_2CO_3; 1·6%, w/v, glycine; 0·045%, w/v, $CuSO_4$) and 1 ml 5 mM-neocuproine (Sigma) were added. Reaction tubes were heated in boiling water for 10 min, the contents were diluted to 10 ml with distilled water and the absorbance was read at 450 nm. One unit of enzyme was defined as that amount which produced an A_{450} of 1 after a 15 min incubation. Estimations of protein concentration were carried out by the method of Bradford (1976).

(ii) *Enzyme purification.* The following buffers were used in the purification procedures. Buffer 1: 50 mM-imidazole/HCl pH 6·5, 1 mM phenylmethylsulphonylfluoride (PMSF), 2% (v/v) glycerol. Buffer 2: buffer 1 containing 0·1 M-NaCl. Buffer 3: buffer 1 containing 1·5 M-NaCl. Buffer 4: 50 mM-Tris pH 7·6, 1 M-KCl, 1 mM-PMSF. Buffer 5: 50 mM-Tris pH 7·6, 1 mM-PMSF. All steps were carried out at 4 °C. SDS-PAGE was done essentially as described by Laemmli (1970) using 12·5% (w/v) polyacrylamide gels.

(iii) *Purification of extracellular agarase.* L-broth filtrates (450 ml) derived from cultures of *S. lividans* TK24(pMT608), *S. lividans* TK24(pIJ2001) and *S. coelicolor* M145 were brought to 75% saturation with ammonium sulphate and the precipitates collected by centrifugation at 16000 ***g*** for 15 min. The pellets were each dissolved in 10 ml of buffer 1 and the suspensions centrifuged at 5000 ***g*** for 5 min to remove particulate matter; the supernatants were adjusted to 15 ml with buffer 1. About 40 ml (settled volume) Bio-gel A-0·5 m was collected by centrifugation for 2 min at 1500 ***g***. Three 10 ml portions of the packed resin were washed twice with buffer 1 and then gently stirred with each of the agarase supernatants for 30 min. The resin was collected by centrifugation and each sample washed with three 10 ml portions of buffer 1. The agarase was eluted from the resin by stirring the beads with three 5 ml portions of buffer 3, each for 5 min. The supernatants were centrifuged briefly to remove remaining agarose beads and the protein was concentrated by dialysis without stirring against 50% (w/v) ammonium sulphate. The resulting protein precipitate was dissolved in 1 ml buffer 1. In a modified procedure a culture filtrate (410 ml) of *S. lividans* TK24(pMT608) was subjected to dialysis without stirring against two 600 ml volumes of 50% (w/v) ammonium sulphate, each for 16–18 h. The protein precipitate was collected by centrifugation, dissolved in 15 ml buffer 2 and applied to a 2·5 × 45 cm Bio-gel P-100 gel filtration column equilibrated with the same buffer. The column was eluted at a flow rate of 10 ml h^{-1} into 1·4 ml fractions; those containing significantly purified agarase (Fig. 4) were subjected to dialysis without stirring and the precipitated protein was dissolved in 10 ml buffer 1. Agarase was further purified by affinity binding to agarose beads as described above.

(iv) *Purification of intracellular agarase.* Mycelium (18 g) of *S. lividans* TK24(pMT608) was washed consecutively with buffers 4 and 5 before resuspension in 20 ml buffer 5. The mycelium was passed through a French press and the extracts were centrifuged for 10 min at 12000 ***g***. The supernatant was adjusted to 35% saturation with ammonium sulphate and the precipitated protein collected by centrifugation. The pellet was dissolved in 4 ml buffer 1 and dialysed against 200 ml of the same buffer for 2 h. Agarase was purified by affinity binding as described above but using 3 ml packed Bio-gel A-0·5 m resin and by eluting with 3 × 2 ml volumes of buffer 1.

In vitro transcription–translation. In vitro transcription–translation studies were carried out as described by Thompson *et al.* (1984) except that 2·8 μg plasmid DNA was used in each assay. SDS-PAGE was done essentially as described by Laemmli (1970) using a 12·5% polyacrylamide gel and fluorography was as described by Bonner & Laskey (1974).

RESULTS

Cloning and expression of the agarase gene in S. lividans 66

Total DNA from *S. coelicolor* M145 was completely digested with *Bam*HI or *Bcl*I and ligated with the plasmid vector pIJ61 (Thompson *et al.*, 1982) that had been cleaved at the unique *Bam*HI site within the neomycin resistance gene and treated with CIAP. The ligation mixtures were used to transform protoplasts of TK24, a derivative of the naturally Dag^- species of *S. lividans* 66. Transformants were selected using the thiostrepton resistance gene of the vector and Dag^+ colonies were recognized by their ability to sink into the surface of the agar plate. Six Dag^+ clones were obtained from total DNA cleaved by *Bam*HI and three from cleavage with *Bcl*I. All agarase-producing clones were thiostrepton-resistant and neomycin-sensitive as expected.

Restriction endonuclease mapping and sub-cloning of dagA

Several Dag^+ isolates derived from digestion of *S. coelicolor* DNA with *Bam*HI or *Bcl*I contained inserts of approximately 5·1 kb or 4·2 kb respectively; the *Bam*HI inserts contained a 4·2 kb *Bcl*I fragment. Given the ease of excision of the cloned fragment from pIJ61, one of the *Bam*HI-derived clones (pIJ2001) was chosen for further study. A restriction endonuclease cleavage map of the inserted fragment is shown in Fig. 1. Sub-cloning of fragments generated by digestion of this segment with *Xho*II into the *Bam*HI site of pIJ61, localized the coding region of *dagA* to a 1·8 kb fragment extending to the right of *Bam*HI site 1 (Fig. 1, hatched bar). The Dag^+ phenotype of the original clones containing the 4·2 kb *Bcl*I fragment (Fig. 1, sites 5–11) permitted further localization to a region extending 1·2 kb to the right of *Bcl*I site 5 (Fig. 1, stippled bar), in good agreement with the results of Kendall & Cullum (1984).

Carbon catabolite repression of the cloned agarase gene

To assess whether the cloned fragment present in pIJ2001 contained regulatory sequences required for carbon catabolite repression, *S. lividans* TK24(pIJ2001), *S. lividans* TK24 and *S. coelicolor* M145 were grown on agar in the presence and absence of different sugars. Agarase activity in both TK24(pIJ2001) and M145 was much reduced in the presence of glucose (Fig. 2)

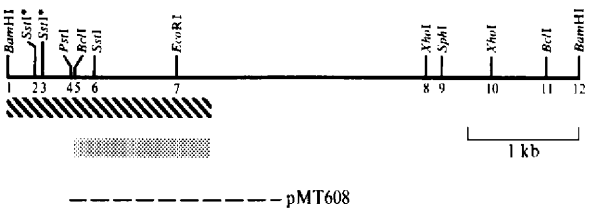

Fig. 1. Restriction map of the 5·1 kb *Bam*HI insert of pIJ2001. The hatched and stippled bars indicate the segments deduced to contain the coding and regulatory region of the agarase gene. The dashed lines indicate the extent of the insert present in pMT605 and pMT608. * Although only one *Sst*I site could be identified at this position by restriction endonuclease mapping, sequence analysis (Buttner *et al.*, 1987) indicated the presence of two sites separated by 49 base pairs. There were no sites for *Bgl*II, *Cla*I, *Hin*dIII, *Xba*I and *Kpn*I.

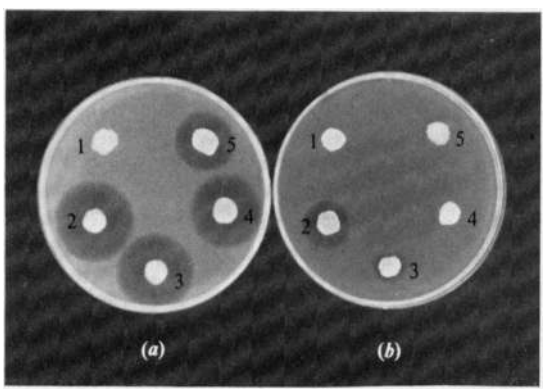

Fig. 2. Carbon catabolite repression of agarase production by *S. coelicolor* M145 and *S. lividans* TK24 transformants containing the cloned agarase gene, (*a*) NMM; (*b*) NMM + glucose. Glucose was used at a final concentration of 5% (w/v), although essentially similar results were obtained at 1% (w/v). Agarase activity results in partial liquefaction and clearing of the agar around each producing strain. 1, TK24; 2, TK24(pMT608); 3, TK24(pMT605); 4, TK24(pIJ2001); 5, M145. Incomplete repression of production by TK24(pMT605) and TK24(pMT608) probably reflects the high copy number of these plasmids and/or transcriptional readthrough from vector promoters.

and, to a lesser extent, in the presence of glycerol, maltose, arabinose or mannitol (data not shown). Similar studies indicated that expression of agarase from the 1·8 kb *Bam*HI–*Xho*II-derived fragment described above (Fig. 1) and from the original 4·2 kb *Bcl*I fragment was also reduced in the presence of glucose. Since the glucose effect did not depend on the orientation of either the 5·1 kb *Bam*HI or 4·2 kb *Bcl*I fragments nor on the restriction site and vector used for cloning (data not shown), these results strongly suggest that the segment extending 1·2 kb to the right of *Bcl*I site 5 contains not only the coding region of *dagA* but also *cis*-acting sequences that permit carbon catabolite repression of agarase production.

Purification of agarase

Preliminary experiments indicated that agarase would bind to agarose in the form of Bio-gel A-0·5 m beads. Attempts were therefore made to use this property for enzyme purification. Culture broths of *S. coelicolor* M145, and of *S. lividans* TK24 containing either pMT608 or pIJ2001, were subjected to ammonium sulphate precipitation and the extracellular agarase activity was purified by affinity binding to agarose beads. A cell-free extract of a culture of *S. lividans* TK24(pMT608) was treated in a similar fashion in an attempt to isolate the intracellular form of the enzyme. Samples of the material were analysed by SDS-PAGE (Fig. 3).

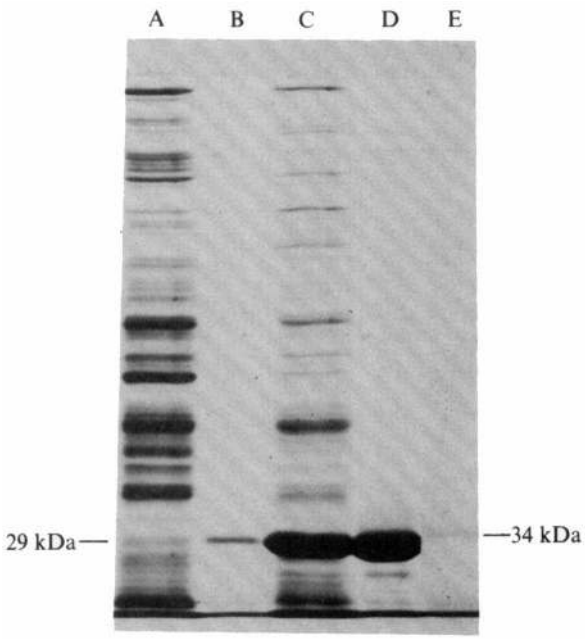

Fig. 3. SDS-PAGE of extracellular and intracellular forms of agarase. Lane A, extracellular protein from *S. coelicolor* M145 after ammonium sulphate precipitation (50 μg); lane B, same as A after affinity purification (5 μg); lane C, extracellular protein from *S. lividans* TK24(pMT608) after ammonium sulphate precipitation (50 μg); lane D, same as C after affinity purification (35 μg); lane E, cell-free extract of *S. lividans* TK24(pMT608) after affinity purification (5 μg). The sizes indicated were estimated from gels (not shown) that gave better resolution of the two forms of agarase.

Table 1. *Yield and purity data for the modified procedure for the purification of extracellular agarase*

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification factor	Yield (%)
Filtrate	410	111	960	8·7	–	100
Ammonium sulphate	15	71·6	1215	17·0	1·96	127
P-100	2·2	5·8	788	135	15·7	82
Affinity	1·1	2·03	616	303	35·1	64

The extracellular form of agarase was provisionally identified as a protein of about 29 kDa (Fig. 3, lanes B, C and D). Under the growth conditions used over 50% of the extracellular protein produced by *S. lividans* TK24(pMT608) appeared to be agarase (lane C); similar amounts were also produced by *S. lividans* TK24 containing either pMT605 or pIJ2001 (data not shown). Given a total extracellular protein concentration of 127 μg ml^{-1}, the amount of agarase in the culture supernatant was estimated by gel scanning to be over 60 μg ml^{-1}; based on a comparison of specific activities, this represents an 860-fold overproduction of agarase compared to M145 grown under similar conditions. The simple affinity binding procedure yielded material which was over 95% homogeneous (lane D; the minor bands below the presumptive agarase band increased in intensity after incubation of the material for prolonged periods at 30 °C, suggesting that they are degradation products of agarase). Furthermore, a presumptive intracellular form could be purified from cell lysates which appeared to be larger than the secreted enzyme by about 5 kDa (lane E; this approximate difference in size was also observed on other gels that gave better resolution of the two forms). These observations suggest that the intracellular form of the enzyme possesses a leader peptide of a similar size to those of other proteins secreted by Gram-positive bacteria (Chang, 1987).

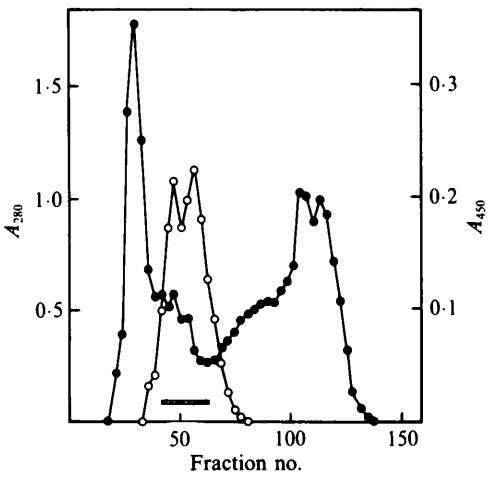

Fig. 4

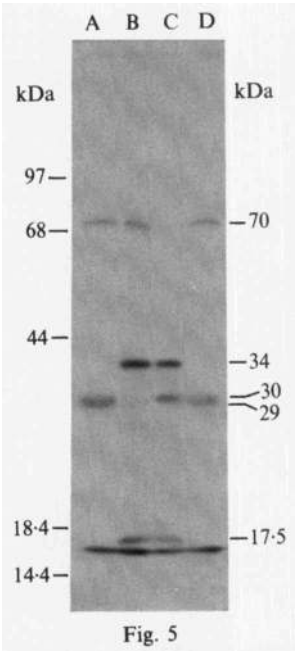

Fig. 5

Fig. 4. Protein elution (●, A_{280}) and agarase activity (○, A_{450}) profile of the ammonium sulphate precipitated filtrate of *S. lividans* TK24(pMT608) after gel filtration on Bio-gel P-100. Samples (50 µl) of every third fraction were assayed for agarase activity. The fractions pooled for subsequent affinity purification are indicated by the hatched bar.

Fig. 5. *In vitro* transcription–translation studies of the cloned agarase gene. Lanes A and D, pIJ702; lane B, pMT608; lane C, pMT605. The size markers were phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), β-lactoglobulin (18·4 kDa) and lysozyme (14·4 kDa).

Subsequent modifications to the purification scheme for extracellular agarase included initial protein precipitation by dialysis without stirring and Bio-gel P-100 chromatography. Yield and purity data on this procedure are shown in Table 1. Agarase eluted in two peaks from the P-100 column (Fig. 4). When individual column fractions corresponding to these peaks were analysed by SDS-PAGE, no differences were observed in the mobility of the agarase band, suggesting that the two peaks, which were observed reproducibly, arose from aggregation of the native protein. SDS-PAGE of the affinity-purified fraction indicated that the enzyme was essentially homogeneous. This modified procedure gave higher yields of agarase than the original method (64% and 4·8% respectively). Much of this increase was due to the inclusion of the Bio-gel P-100 column, which significantly increased the yield of enzyme obtained from the affinity binding step, presumably by removing substances which interfered with binding of agarase to the agarose beads.

Although cultures of *S. lividans* TK24 containing either pMT605 or pIJ2001 produced consistently high yields (45–60 µg ml^{-1}), the amount of agarase secreted by cultures of TK24(pMT608) varied considerably, from about 7 µg ml^{-1} to about 60 µg ml^{-1}. Much of this variation probably stems from structural instability which was observed in some preparations of pMT608 DNA (M. J. Buttner, unpublished results).

In vitro transcription–translation of the dagA gene

In an attempt to confirm the presence of a pre-processed form of agarase, samples of pMT605 and pMT608 DNA (Kendall & Cullum, 1984) were used to prime synthesis of ^{35}S-labelled proteins in a *S. coelicolor* coupled *in vitro* transcription–translation system (Thompson *et al.*, 1984). Each clone yielded a protein of about 34 kDa (Fig. 5) which was not observed in extracts primed by pIJ702 alone and whose size was in good agreement with that of the presumptive

intracellular form of the enzyme described earlier. This protein also bound selectively to agarose beads using the affinity binding procedure (data not shown). An additional protein band of about 17·5 kDa and of unknown function was also produced from both pMT605 and pMT608.

Some properties of the purified enzyme

pH optimum studies on the purified enzyme indicated that agarase was active over a broad range of pH values (5–8·5) in acetate or imidazole buffers, with a peak of activity at pH 6. While the enzyme was active in Tris buffers, the level of activity was significantly lower than that observed in imidazole and the pH optimum was shifted to 7·5. The enzyme activity was not stimulated by Ca^{2+}, Mg^{2+} or Mn^{2+}. Indeed, at higher concentrations (10–20 mM) Ca^{2+} and Mn^{2+} (but not Mg^{2+}) inhibited the activity of the enzyme.

DISCUSSION

The agarase gene (*dagA*) of *S. coelicolor* was cloned and the coding sequence localized to a 1·2 kb region. Expression of the cloned gene is subject to carbon catabolite repression, implying that the regulatory region of *dagA* is also present in the cloned fragment.

A simple agarase purification procedure was developed, based on the affinity of the enzyme for agarose, which permits the protein to be purified to near homogeneity. Comparison of the M_r of the purified extracellular protein with that of the purified intracellular form and the *in vitro* transcription–translation product of *dagA* implies that agarase is synthesized as a pre-protein possessing a signal peptide which is removed during secretion into the extracellular medium. This suggestion is further supported by the recent determination of the nucleotide sequence of *dagA* (Buttner *et al.*, 1987). The *in vitro* transcription–translation experiments also suggest that a 17·5 kDa protein is encoded by a gene adjacent to *dagA*.

Given the potentially useful regulatory features of *dagA*, and that agarase constitutes over half of the extracellular protein produced by some of the clones described here, *dagA* may be particularly suitable for the development of expression–secretion vectors for these industrially familiar bacteria. In addition to making translational fusions directly to the signal peptide, it would also seem appropriate to consider using the affinity purification procedure to isolate, as fusion proteins, foreign gene products that have been fused to the carboxy-terminal end of the agarase, providing that such proteins retain the ability to bind to agarose.

This work has provided a basis for the further analysis of the structure and regulation of the genes responsible for agar utilization by *S. coelicolor* A3(2) (Buttner *et al.*, 1987). The resulting material will be used to study the molecular events involved in the induction of *dagA* and, since the cloned gene is also subject to glucose repression, to study the mechanism of carbon catabolite repression in *Streptomyces*.

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