A Non-specific Deoxyribonuclease with Restriction Function in *Streptomyces glaucescens*

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A non-specific deoxyribonuclease with a possible role in the restriction of some actinophages was detected in *Streptomyces glaucescens* ETHZ 22794. Production of this enzyme activity was influenced by the medium composition, indicating nutritional control of enzyme synthesis. Restriction was confirmed when phage adsorption and efficiency of plating in nuclease-productive and non-productive media were investigated, and also by analysis of a mutant which lacked exonucleolytic activity. *In vivo* escape from restriction in nuclease-productive media is mainly related to the ability of phages to adsorb in a growth phase earlier than that in which enzyme synthesis occurs.

INTRODUCTION

A significant number of type II endonucleolytic activities have been detected in *Streptomyces* spp. (Roberts, 1984). The current belief is that these enzymes are the biochemical instruments of biological restriction–modification (RM) systems, available in the same strains. However, in only a few cases has it been demonstrated that cellular endonuclease activity is the cause of biological restriction (Chater & Wilde, 1976; Chater & Carter, 1979; Voeykova *et al.*, 1980; Klaus *et al.*, 1981). Nevertheless, nucleolytic activities, including exocellular deoxyribonucleases requiring Mg^{2+} ions, are generally accepted to be widespread in streptomycetes (Yanagida & Ogawara, 1980; Chater & Hopwood, 1984). In addition to a hypothetical nutritional role for these enzymes, their possible implications for other processes such as recombination, conjugation and transformation, and DNA replication and repair have not been discarded (Linn, 1982). No genetic studies have been realized in most cases, although they would be interesting from both academic and applied points of view (Chater & Hopwood, 1984).

Type I or III endonuclease activities have yet to be described in *Streptomyces*, and *S. glaucescens* has no detectable type II endodeoxyribonucleolytic activity in crude cell-free extracts (J. Sánchez, unpublished observations). Phage and plasmid restriction, in the absence of detectable type II restriction enzymes, has also been noted in some *Streptomyces* species (Chater & Hopwood, 1984; De los Reyes-Gavilán *et al.*, 1988). The major DNAase activity detected in the *S. glaucescens* strain studied here is a unique non-specific deoxyribonuclease located outside the cellular cytoplasm in the cell wall-membrane region (J. F. Aparicio and others, unpublished). In this report, we show that the *S. glaucescens* nuclease, whose synthesis is under nutritional control, has a role in phage restriction.

METHODS

Bacterial and phage strains. *Streptomyces glaucescens* ETHZ 22794 and the derived DNAase[–] mutant GLO 86 were used. Characteristics, source and hosts of phages P17 and P27 were described by Sánchez *et al.* (1985). Phage ϕ A7 was isolated directly from soil on *S. antibioticus* ATCC 11891 (M. R. Rodicio & L. A. Diaz, unpublished results).

Growth media. Growth and sporulation of bacterial strains were achieved on GAE medium at 30 °C (Hardisson *et al.*, 1978). Phages were grown on bacteria cultured on Oxoid nutrient agar supplemented with 0.5% (w/v) glucose (NAG medium) and on solid CC medium with adequate Ca^{2+} and Mg^{2+} concentrations (Sánchez *et al.*, 1985). In the case of ϕA7 , 100 mM- Ca^{2+} and 10 mM- Mg^{2+} were used. These media without agar were used to grow strains in liquid culture. Additional enzymic and phage growth tests were also done in minimal medium (MM) which contained 1% (w/v) glucose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05% K_2HPO_4 with either 0.1% Oxoid acid casein hydrolysate or 0.1% asparagine as nitrogen source. The medium used to select mutants lacking DNAase activity (TBS medium) comprised 1% (w/v) glucose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% K_2HPO_4 and 1.5% (w/v) agar. After sterilization, 0.1 mg toluidine blue ml^{-1} and 1 mg herring sperm DNA (Boehringer) ml^{-1} were added, before pouring plates.

Propagation and efficiency of plating (e.o.p.) of the phages. Titres of phage suspensions and e.o.p. values were obtained in the different media, as described previously (Sánchez *et al.*, 1985). All incubations were at 30 °C.

Adsorption studies. The adsorption capacity of the phages to bacteria grown in CC or nutrient broth/glucose (NBG) medium was measured using the method described by De los Reyes-Gavilán *et al.* (1988).

Measurement of growth. The method of Braña *et al.* (1985), based on homogenization of mycelial suspensions by ultrasonic treatment and measurement of optical density at 600 nm, was used for quantification of growth in liquid media.

DNAase assay in cell-free extracts. Synthesis of enzyme during growth was determined by analysing supernatants prepared by high-speed centrifugation of sonicated mycelial pellets.

Sonication was carried out at 14 μm frequency for 30 s periods, until complete rupture, in an extraction buffer which contained 50 mM-Tris/HCl (pH 7.5), 0.1 mM-phenylmethylsulphonyl fluoride (PMSF), 7 mM-2-mercaptoethanol, 1 mM-EDTA and 200 mM-NaCl. The ratio of weight of mycelium sample to volume of extraction buffer was constant in all determinations. The ruptured mycelium was ultracentrifuged (39 200 g for 1 h at 4 °C) and the supernatant used as the source of enzyme.

The enzyme assay was done using λ DNA (0.5 μg) as substrate and 3 μl of enzyme preparation in a final volume of 30 μl of reaction buffer (10 mM-Tris/HCl pH 7.5, 10 mM- MgCl_2 , 20 mM-NaCl, 1 mM-dithiothreitol) for 60 min at 37 °C. After stopping the reaction (Sánchez *et al.*, 1985), 30 μl samples were electrophoresed on 0.8% (w/v) agarose gels, stained with ethidium bromide (1 $\mu\text{g ml}^{-1}$) and observed under UV light.

Partial purification of the DNAase. *S. glaucescens* DNAase was partially purified from mycelium grown for 24 h in NBG medium. All the steps were carried out at 4 °C. The mycelium (50 g wet weight) was washed and resuspended in 200 ml of a solution which contained 10 mM-phosphate buffer (pH 7.0), 7 mM-2-mercaptoethanol, 1 mM-EDTA, 0.1 mM-PMSF, 10% (v/v) glycerol and 400 mM-NaCl. The suspension was sonicated and centrifuged as described above. The NaCl concentration was adjusted to 200 mM and the solution chromatographed through a 160 ml phosphocellulose column. Flow rate was adjusted to 20 $\text{ml h}^{-1} \text{cm}^{-2}$, and the fractions eluted with a 1000 ml NaCl gradient (200–700 mM) in the former buffer. Active fractions, eluting at 0.35 M-NaCl, were pooled, dialysed against 20 mM-Tris/HCl (pH 7.5) plus the compounds specified above (except NaCl) and chromatographed through a 50 ml column of DEAE-cellulose (DE-52, Whatman). Active fractions eluted at 0.1 M-NaCl. The concentrated peak of activity was used as the source of enzyme in phage DNA digestion experiments. Phage DNAs were isolated by the method of Chater *et al.* (1982).

Isolation of mutants lacking nucleolytic activity (DNAase⁻). Mutagenesis was carried out on a spore suspension of *S. glaucescens* by irradiation under UV light (254 nm) for 7 min. Plates of GAE medium were inoculated with the irradiated spore suspension, and incubated at 30 °C in complete darkness until sporulation occurred. Mutant selection was carried out by velvet replication to plates of TBS medium. The method is based on the hydrolysis of DNA by the nuclease and disruption of the DNA–toluidine blue complex (Roganti & Rosenthal, 1983), so that mutant colonies, by contrast with the wild-type, lack a clear halo. Plates were incubated at 30 °C and examined after 48 h. Possible mutants were confirmed by a nuclease assay on cell-free extracts.

RESULTS

Influence of medium composition on enzyme production

Crude cell-free extracts of *S. glaucescens* grown on two different media were tested for the presence of nuclease activity, using λ DNA as substrate. No activity was detected in cultures grown in CC medium until after 48 h incubation, when slight activity was detected (Fig. 1c). On NBG medium activity was produced after 21 h, towards the end of the exponential phase of growth, and was greater (Fig. 1b). As the growth curves were similar in both media (Fig. 1a), we can infer that medium composition influences production of the enzyme. This was corroborated when other media were tested: in MM with casein hydrolysate or asparagine as nitrogen source no activity was observed at any point in the growth cycle (data not shown).

Fig. 1. Growth curves and presence of DNAase activity in cell-free extracts of *S. glaucescens* wild-type and DNAase⁻ mutant strains. (a) Growth of the wild-type in NBG medium (△) and in CC medium (▲). (b) Activity on λ DNA (0.5 μg) of cell-free extracts of wild-type mycelium grown in NBG medium for 13 h, 17 h, 21 h, and 48 h. (c) As for (b) except that the mycelium was grown in CC medium for 13 h, 17 h, 21 h and 48 h. (d) Activity on λ DNA (0.5 μg) of cell-free extracts of mycelium from the DNAase⁻ mutant grown in NBG medium for 24 h and 48 h.

Growth efficiency of actinophages on the wild-type and GLO 86 mutant of S. glaucescens

The rate of halo formation on TBS agar, indicative of DNAase activity, varied amongst the large number of colonies examined. The crude cell-free extracts of NBG cultures derived from colonies lacking a halo were tested for the presence of nuclease activity, using λ DNA as substrate. This resulted in the selection of a mutant (GLO 86) lacking the DNAase activity (DNAase⁻; Fig. 1 d), and several which exhibited reduced enzyme production (data not shown).

Two virulent (P17 and P27) and one temperate (φA7) actinophages were selected to test the role of the DNAase of *S. glaucescens* in restriction. For this purpose, the e.o.p. of the phages on wild-type and mutant strains was investigated in nuclease-productive and non-productive media (Table 1). With the wild-type, phage P27 showed a significantly lower e.o.p. on NAG (highly productive medium) than on CC. With the DNAase⁻ mutant, the e.o.p. on NAG medium was significantly higher than for the wild-type. In addition, plaque sizes obtained on the wild-type

Fig. 2. Characteristics of lytic plaques of $\phi A7$ on *S. glaucescens* wild-type and DNAase⁻ mutant strains. All plates were incubated for 48 h. (a, b) $\phi A7$ on wild-type on NAG medium (a) and on CC medium (b); (c, d) $\phi A7$ on DNAase⁻ mutant on NAG medium (c) and on CC medium (d).

Table 1. Plating efficiency of three actinophages on wild-type *S. glaucescens* ETHZ 22794 and its DNAase⁻ mutant

Phages were tested as high-titre suspensions obtained from the original host. The e.o.p. values, calculated after incubation of plates for 30 h, are referred to those obtained in the original host (see Table 2). Values are the means of at least three separate experiments.

Phage	Wild-type		DNAase ⁻ mutant	
	NAG medium	CC medium	NAG medium	CC medium
P17	3.1×10^{-3}	2.5×10^{-3}	4.0×10^{-3}	3.9×10^{-3}
P27	1.1×10^{-3}	1.32	1.9×10^{-1}	1.87
$\phi A7$	$< 2.1 \times 10^{-8}$	$< 8.6 \times 10^{-7}$	2.5×10^{-3}	2.0×10^{-3}

strain were smaller (approx. 1 mm diameter) than those obtained on the DNAase⁻ mutant (approx. 1.5 mm diameter) on both media. Remarkably, $\phi A7$ was unable to produce visible plaques on the wild-type strain on either of the media tested, but grew and gave similar e.o.p. values (Table 1) and plaque sizes (Fig. 2) in the DNAase⁻ mutant on both media. More significantly, $\phi A7$ grew efficiently on the wild-type on MM with casein hydrolysate or asparagine (e.o.p. 0.15 and 0.1 respectively), where the enzyme is not produced at all throughout the growth cycle. Phage P17 gave similar e.o.p. values on the different media and strains tested, and there were no significant differences in the size of the plaques on each.

The exocyttoplasmic location of the DNAase of *S. glaucescens* (see Introduction) implies that the existence of an accompanying modification system in the cell, which could otherwise protect the host DNA from degradation, is unnecessary. If this is true, P27 grown in the wild-type, and $\phi A7$ in the DNAase⁻ mutant, should be unmodified after passage through *S. glaucescens*. To check this, e.o.p. measurements and *in vitro* digestion of phage DNA by the purified enzyme were carried out. Phages P27 and $\phi A7$ obtained from the wild-type or the DNAase⁻ mutant, respectively, showed identical e.o.p. values to those presented in Table 1. Furthermore, samples of DNA isolated from P27 and $\phi A7$ grown in the wild-type and the DNAase⁻ mutant were

Fig. 3. Hydrolysis of phage DNA by the partially purified DNAase from *S. glaucescens*. Reactions were carried out for 2 h as described in Methods. The lanes contain: undigested P27 DNA obtained from *S. coelicolor* ETHZ A3170 (original host) (1) and from *S. glaucescens* (3); P27 DNA from these sources digested with *S. glaucescens* nuclease (2 and 4 respectively); undigested ϕ A7 DNA obtained from *S. antibioticus* (5) and from the *S. glaucescens* DNAase⁻ mutant (7); ϕ A7 DNA from these sources digested with *S. glaucescens* nuclease (6 and 8 respectively).

Table 2. Adsorption of phages to *S. glaucescens* wild-type and DNAase⁻ mutant

Suspensions of phages were obtained from the original hosts: P17, *S. albus* GJ 1074; P27, *S. coelicolor* ETHZ A3170; ϕ A7, *S. antibioticus* ATCC 11891. The values are expressed as percentage decrease of free phage with respect to the control (original host) value of 100% after 120 min incubation (60 min in the case of ϕ A7). The adsorption efficiency was measured using mycelium from early exponential phase (square brackets), mid-exponential phase (round brackets) or early stationary phase (no brackets). Values are the means of three determinations.

Phage	Wild-type			DNAase ⁻ mutant	
P17	—	(33)	8	(35)	5
P27	[2]	(85)	69	(86)	62
ϕ A7	[0]	(0)	54	(8)	60

sensitive to hydrolysis by the partially purified nuclease (Fig. 3). All of these results clearly show the absence of a DNA modification system protecting phage DNA from hydrolysis in *S. glaucescens*.

Adsorption studies

Lack of specific receptors in the cell wall of *Streptomyces* spp. may reduce the access of the phages and result in low e.o.p. or even total absence of infection. To distinguish this phenomenon from actual restriction, we studied the adsorption capability of the phages to the wild-type and the DNAase⁻ mutant of *S. glaucescens* in both NAG and CC medium. As the values were similar, only those results obtained in NAG medium are presented in Table 2.

Phage P17 (with similar e.o.p. values on both media and hosts) gave an adsorption efficiency to the wild-type and DNAase⁻ strains of *S. glaucescens* lower than 36% of the value obtained in the control (original host). In this case, the e.o.p. values observed are most likely due to limited adsorption capability, since the phage was unable to adsorb during the stationary phase, when the enzyme is produced (Table 2).

Phage P27 showed efficient adsorption in both mid-exponential and early stationary phase, thus suggesting that phage restriction and not adsorption incapacity was responsible for the low e.o.p. values obtained in NAG medium with the wild-type strain. This restriction phenomenon was confirmed when adsorption of ϕ A7 was tested. In this case, the phage was able to adsorb to wild-type *S. glaucescens* (in which it was unable to grow), and to the DNAase⁻ mutant, but only in the stationary growth phase (Table 2), during which enzyme is produced. Percentage values are overrepresented with respect to the other phages, due to the fact that adsorption of ϕ A7 to the original host (*S. antibioticus*) is itself somewhat less efficient than, for example, adsorption of P27 to its host (*S. coelicolor* ETHZ A3170; Sánchez *et al.*, 1985). Thus adsorption inefficiency in the growth phase where the enzyme still was not produced, together with the restriction activity of the DNAase in a later phase when the phage adsorbs, explain the absence of visible plaques of ϕ A7 on wild-type *S. glaucescens* in CC medium. The low adsorption ability of ϕ A7 is reflected in the relatively low e.o.p. reached in the DNAase⁻ mutant on both media (Table 1).

DISCUSSION

The most obvious approach for detecting *in vivo* restriction in *Streptomyces* spp. is to use actinophages as biological indicators (Chater, 1978), and thus we have used several actinophages to detect *in vivo* restriction in *S. glaucescens* ETHZ 22794. This strain harbours a non-specific DNAase activity which, after purification, is able to degrade linear and circular DNA substrates (J. Sánchez, unpublished observations).

All three phages tested, including those which produce a high e.o.p. in *S. glaucescens*, are sensitive *in vitro* to the action of the nuclease, thus suggesting that a different mechanism operates *in vivo* to avoid, at least partially, the action of the enzyme in a productive medium. No modifying activity has been detected in this strain; thus, a simple explanation for the *in vivo* escape would be the existence of adsorption capability in a growth phase in which the enzyme is not produced. In this way, the different e.o.p. values observed in the nuclease-productive medium should be determined by two factors: first, the adsorption capability of phages in a more or less wide phase of host growth period, and second, the presence of the nuclease in NAG medium at the end of the exponential phase of growth. For ϕ A7, which adsorbs mainly in this phase, results corroborated that supposition, as no plaques were observed on the productive media (NAG or CC) but plaque formation was efficient on non-productive media (MM with casein hydrolysate or asparagine). Phage P27 adsorbs at the mid-exponential growth phase where the enzyme is not yet produced, thus explaining the ability of this phage to produce lytic plaques in *S. glaucescens* on CC medium.

In the case (DNAase⁻ mutant) where we have excluded the enzymic factor, phage behaviour is determined only by adsorption capability, thus constituting a very suitable system to study restriction in this strain. The e.o.p. values obtained with ϕ A7 and P27 in the DNAase⁻ mutant, when considered with the relatively efficient adsorption, provide further evidence of the existence of a biological restriction phenomenon in *S. glaucescens*. Phage ϕ A7 is unable to grow on wild-type *S. glaucescens* in either of the two media, and P27 gives a significantly lower e.o.p. on NAG medium (highly productive medium); this phenomenon is attributable to the DNAase since no other nucleolytic activity was detected in this strain.

The production of the enzyme in different growth phases of *S. glaucescens*, and the lack of activity on some media, indicate the involvement of nutritional control in synthesis of the enzyme. This is conceivably related to other possible function(s) in the cell, in addition to its involvement in phage restriction. One possible role, which deserves further study, is catabolic degradation of DNA.

The results are remarkably similar to data obtained in *S. antibioticus*, in which an endodeoxyribonuclease, also subject to nutritional control, and located between the cytoplasmic membrane and the cell wall, fulfils a similar function in phage restriction (De los Reyes-Gavilán *et al.*, 1988). This striking functional analogy opens interesting perspectives on the possible evolutionary relationship between these enzymes and the more 'orthodox' type II restriction endonucleases. A wider study, in which the presence and role of non-specific DNAases is being

analysed in other *Streptomyces* strains containing one or more restriction endonucleases, is in progress. Analysis of the possible influence of the *S. glaucescens* exonuclease and related enzymes on phenomena such as plasmid transformation, transfection and recombination will be of considerable practical interest.

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REFERENCES

- BRAÑA, A. F., WOLFE, S. & DEMAİN, A. L. (1985). Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. *Canadian Journal of Microbiology* **31**, 736–743.
- CHATER, K. F. (1978). Restriction in *Streptomyces*. In *Nocardia and Streptomyces; Proceedings of the International Symposium on Nocardia and Streptomyces*, pp. 303–311. Edited by M. Mordarski, W. Kurylowicz & J. Jeljaszewicz. Stuttgart & New York: Gustav Fischer Verlag.
- CHATER, K. F. & CARTER, A. T. (1979). A new, wide host-range, temperate bacteriophage (R4) of *Streptomyces* and its interaction with some restriction-modification systems. *Journal of General Microbiology* **115**, 431–442.
- CHATER, K. F. & HOPWOOD, D. A. (1984). *Streptomyces* genetics. In *The Biology of the Actinomycetes*, pp. 229–286. Edited by M. Goodfellow, M. Mordarski & S. T. Williams. London: Academic Press.
- CHATER, K. F. & WILDE, L. C. (1976). Restriction of a bacteriophage of *Streptomyces albus* G involving endonuclease *SalI*. *Journal of Bacteriology* **128**, 644–650.
- CHATER, K. F., HOPWOOD, D. A., KIESER, T. & THOMPSON, C. J. (1982). Gene cloning in *Streptomyces*. *Current Topics in Microbiology and Immunology* **96**, 69–95.
- DE LOS REYES-GAVILAN, C. G., APARICIO, J. F., BARBES, C., HARDISSON, C. & SANCHEZ, J. (1988). An exocyttoplasmic endonuclease with restriction function in *Streptomyces antibioticus*. *Journal of Bacteriology* **170**, 1339–1345.
- HARDISSON, C., MANZANAL, M. B., SALAS, J. A. & SUAREZ, J. E. (1978). Fine structure, physiology and biochemistry of arthrospore germination in *Streptomyces antibioticus*. *Journal of General Microbiology* **105**, 203–214.
- KLAUS, S., HARTMANN, M., KRUGEL, H., ROTH, M., WALTER, F., RAUTENSHEIN, Y. I. & SOLOV'eva, N. Y. (1981). Restriction of *Streptomyces* phage SH5 by endonuclease *ShyI* from *Streptomyces hygroscopicus* 0477. *Molecular and General Genetics* **184**, 286–288.
- LINN, S. M. (1982). Nucleases involved in DNA repair. In *Nucleases*, pp. 59–83. Edited by S. M. Linn & R. J. Roberts. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- ROBERTS, R. J. (1984). Restriction and modification enzymes and their recognition sequences. *Nucleic Acids Research* **12** (Supplement), r167–r204.
- ROGANTI, F. S. & ROSENTHAL, A. L. (1983). DNAases of *Acholeplasma* spp. *Journal of Bacteriology* **155**, 802–805.
- SANCHEZ, J., BARBES, C., HERNANDEZ, A. DE LOS REYES-GAVILAN, C. R. G. & HARDISSON, C. (1985). Restriction-modification systems in *Streptomyces antibioticus*. *Canadian Journal of Microbiology* **31**, 942–946.
- VOEYKOVA, T. A., OVEKHOV, A. V. & REBENTISH, B. A. (1980). New approaches to the study of restriction and modification systems in actinomycetes. *Actinomycetes* **15**, 152–166.
- YANAGIDA, T. & OGAWARA, H. (1980). Deoxyribonucleases in *Streptomyces*. *Journal of Antibiotics* **33**, 1206–1207.