Characterization of a *Streptomyces-lividans*-type site-specific DNA modification system in the avermectin-producer *Streptomyces avermitilis* permits investigation of two novel giant linear plasmids, pSA1 and pSA2

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The degradation of *Streptomyces avermitilis* DNA samples analysed by conventional pulsed-field gel electrophoresis was shown to be due to Tris-dependent, double-strand cleavage. Using alternative electrophoretic conditions, separation of intact DNA molecules was achieved, permitting the identification of two novel giant linear plasmids: the 100 kb pSA1 and 250 kb pSA2. Use of pSA2 DNA as a probe showed that pSA1 does not cross-hybridize, indicating that the plasmids are not closely related. The site-specificity of the DNA modifications, which render the DNA susceptible to Tris-dependent cleavage, was found to be essentially identical to that of similar modifications found in the DNA of *S. lividans*.

Keywords: Linear plasmids, DNA modification, *Streptomyces avermitilis*.

INTRODUCTION

Among prokaryotes, *Streptomyces* are unusual in that they often contain giant linear plasmids refractive to conventional separation techniques. With the application of pulsed-field gel electrophoresis (PFGE), it became possible to physically characterize examples of these plasmids, some of which had been surmised to exist due to their ability to promote conjugation and chromosomal recombination (Kinashi *et al.*, 1987). The paradigm is the 350 kb linear plasmid, SCP1 of *S. coelicolor* A3(2), which, as well as encoding fertility functions, contains the genes for methylenomycin biosynthesis and resistance. Genetic evidence for this plasmid was reported by Vivian (1971), although physical isolation using PFGE was described only more recently (Kinashi & Shimaji-Muruyama, 1991).

Another conjugative plasmid, SLP2 of *S. lividans* (Hopwood *et al.*, 1983), initially proved impossible to isolate even by application of PFGE. *S. lividans* possesses a site-specific DNA modification system which renders its DNA susceptible to double-strand scission during electrophoresis in Tris buffers (Ray *et al.*, 1992; Zhou *et al.*, 1988). As a consequence, extensive degradation of the DNA occurs during PFGE, so that neither chromosomal nor plasmid DNA can be resolved as discrete molecules. To visualize SLP2, it was necessary first to transfer the

plasmid either to heterologous hosts which do not modify DNA or to a non-modifying mutant, prior to preparing samples for PFGE (Chen *et al.*, 1993). However, the 50 kb linear plasmid could also be resolved by PFGE of wild-type *S. lividans* DNA by changing the electrophoretic conditions; either by substituting HEPES for Tris in buffers used for sample preparation and electrophoresis, or by including a reducing agent in a Tris-based electrophoresis buffer (Evans & Dyson, 1993). Thiourea, for example, can reduce the reactive oxidative Tris derivative, formed at the anode during electrophoresis, which causes double-strand cleavage of the DNA (Ray *et al.*, 1992; T. Ray & P. Dyson, unpublished).

S. avermitilis produces an important series of 16-membered macrocyclic lactones known as avermectins which are potent antiparasitic compounds with broad-spectrum activity against nematode and arthropod parasites (Burg *et al.*, 1979). A natural fertility system has been used to demonstrate linkage between avermectin biosynthetic genes, although it was not possible to associate chromosomal recombination with plasmid fertility functions as is the case for both SCP1 in *S. coelicolor* and SLP2 in *S. lividans* (Ikeda *et al.*, 1987).

Attempts to physically characterize the genome of *S. avermitilis* by PFGE have been thwarted as the DNA is prone to degradation (Kieser *et al.*, 1992). In this study we

have demonstrated that this degradation is attributable to modification of the DNA, similar to that occurring in *S. lividans*, rendering it susceptible to Tris-dependent cleavage during electrophoresis. Hence, using non-degradative PFGE conditions already determined in our laboratory (Evans & Dyson, 1993), we have now succeeded in identifying and investigating two giant linear plasmids present in *S. avermitilis*.

METHODS

Bacterial strains, plasmids and culture conditions. *Streptomyces* strains and plasmids are listed in Table 1. Cultures were grown on GHM agar (glucose, 4 g l⁻¹; yeast extract, 4 g l⁻¹; malt extract, 10 g l⁻¹, CaCO₃, 2 g l⁻¹; agar 15 g l⁻¹, pH 7.2) and, for liquid cultivation, in tryptic soy medium (Dyson & Schrepf, 1987). Plasmid-curing was achieved by nitrosoguanidine mutagenesis (Hopwood *et al.*, 1985) using conditions permitting 10% survival.

Preparation of inserts and pulsed-field gel electrophoresis.

To prepare gel inserts, mycelia were harvested by centrifugation from 3 or 4-d-old liquid cultures. The procedure subsequently followed was originally developed to prepare gel inserts of *S. lividans* DNA, and differs from standard protocols in that for all buffers Tris was replaced by HEPES (Evans & Dyson, 1993). Standard methods were used to prepare inserts containing yeast [*Saccharomyces cerevisiae* K107 (Nasmyth, 1982)] chromosomes or bacteriophage lambda concatamers (Sambrook *et al.*, 1989). PFGE was carried out using a Bio-Rad contour-clamped homogeneous electric field (CHEF-DRII) system. Pulse times were 60 s for 18 h, followed by 90 s for 5 h at 150 V, with a running temperature of 14 °C for gels run in 0.5 × TBE (Sambrook *et al.*, 1989). Alternatively, gels were prepared and run in 1 × HEPES (16 mM HEPES/NaOH pH 7.5, 16 mM sodium acetate, 0.8 mM EDTA) using similar pulse times, unless otherwise stated, at 130 V, again with a running temperature of 14 °C.

DNA manipulations. *Streptomyces* plasmid DNA isolation was

based on an alkaline lysis method (Hopwood *et al.*, 1985). Restriction enzymes were purchased from Gibco-BRL, and digests performed as recommended by the manufacturer. DNA was isolated from gel slices by agarose digestion with GELase (Epicenter Technologies), and subsequent ethanol precipitation.

Transformation procedures. Plasmid DNA was introduced into *S. avermitilis* by polyethylene-glycol-mediated transformation of protoplasts which were subsequently regenerated on RM14 medium (MacNeil & Klapko, 1987). Transformants were selected and subsequently maintained by growth on media containing 10 µg thiostrepton ml⁻¹. Preparation and transformation of *S. lividans* protoplasts were done as described by Hopwood *et al.* (1985), transformants being selected and maintained on media containing 25 µg thiostrepton ml⁻¹.

Southern hybridization. After electrophoretic separation in agarose, DNA was transferred to Hybond N nylon membrane (Amersham) using a vacuum blotting system (Pharmacia or Stratagene). Probes labelled with either [α -³²P]dCTP or digoxigenin-11-dUTP were prepared by random priming using a kit from Boehringer Mannheim. Radiolabelled probes were hybridized to membranes in a solution of 5 × SCP (20 × SCP: 2 M NaCl, 0.6 M Na₂HPO₄, 0.2 M Na₂EDTA), 10% (w/v) dextran sulphate (Pharmacia), 1% (w/v) N-lauroylsarcosine (Sigma), and 1 mg heparin (Sigma) ml⁻¹ at 65 °C and washed twice with 2 × SCP, 0.1% SDS at 65 °C and twice with 0.2 × SCP, 0.1% SDS at 65 °C prior to autoradiography. For digoxigenin-labelled probes, hybridization conditions and subsequent detection by colour reaction with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were according to the manufacturer's instructions (Boehringer Mannheim).

Tris-dependent site-specific DNA cleavage. Plasmid DNA, previously digested with restriction enzymes, was purified by phenol/chloroform extraction and ethanol precipitation. The DNA was redissolved in 10 µl of water to give a concentration of 1 µg ml⁻¹, and then divided equally between two tubes prior to induction of Tris-dependent strand cleavage. A 500 µl vol. of non-activated 1 × TAE buffer (40 mM Tris, 20 mM sodium acetate, 0.8 mM EDTA, pH 7.5 adjusted with acetic acid), maintained at 37 °C, was added to one tube, and 500 µl of activated buffer, again maintained at 37 °C and sampled from 5 mm adjacent to the anode of a horizontal gel chamber after applying a constant voltage of 80 V for 10 min (Ray *et al.*, 1992), was added to the second tube. Both tubes were incubated at 37 °C for 2 h prior to terminating cleavage by addition of 50 µl 3 M sodium acetate, pH 5, and 550 µl propan-2-ol. The DNA was precipitated, pelleted and redissolved prior to gel loading. DNA fragments were fractionated on a 0.9% agarose gel run in 1 × HEPES buffer, prior to Southern hybridization.

RESULTS AND DISCUSSION

PFGE of *S. avermitilis* DNA permits identification of two linear plasmids

DNA prepared from *S. coelicolor* A3(2) could be resolved by PFGE in 0.5 × TBE to give the characteristic profile of presumed circular chromosomal DNA retained in the well, linear chromosomal DNA, estimated at 8 Mb (Kieser *et al.*, 1992) just entering the gel, and the 350 kb SCP1 clearly resolved from the chromosomal DNA (Fig. 1a). However, DNA prepared from either *S. lividans* or *S. avermitilis* appeared as degraded fragments of 100 kb or

Table 1. *Streptomyces* strains

Strain	Plasmids	Source (alternative designation)
<i>S. avermitilis</i> 12804	pSA1, pSA2	NCIMB* (ATCC 31267)
<i>S. avermitilis</i> 31272	pSA1, pSA2	ATCC
<i>S. avermitilis</i> 31272 sc2	pSA1	This study†
<i>S. coelicolor</i> A3(2)	SCP1, SCP2	D. A. Hopwood, John Innes Institute, UK
<i>S. lividans</i>	SLP2, SLP3	D. A. Hopwood, John Innes Institute, UK (1326, 'strain 66')

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† A single colony isolated after nitrosoguanidine mutagenesis of strain 31272.

Fig. 1. Identification of *Streptomyces* linear plasmids by pulsed-field gel electrophoresis. Inserts prepared from *S. coelicolor* A3(2) (lane 1), *S. lividans* (lane 2), *S. avermitilis* 12804 (lane 3) and *Saccharomyces cerevisiae* K107 (lane 4) were electrophoresed in 0.5×TBE (a) and 1×HEPES (b) buffers. Linear plasmids present in each *Streptomyces* species are starred (see text for details). The sizes (kb) of the resolved yeast chromosome markers are indicated.

less in gels run in 0.5×TBE, preventing identification of extrachromosomal elements.

In contrast, DNA from the same gel inserts prepared from all three species could be effectively resolved by PFGE in 1×HEPES buffer, revealing the 50 kb SLP2 linear plasmid in *S. lividans*, and two extrachromosomal elements estimated at 250 kb and 100 kb in *S. avermitilis* 12804 (Fig. 1b). To investigate the topology of the latter two elements, separate gels were run for different pulse times in 1×HEPES. During PFGE the extent of migration of circular molecules relative to linear molecules varies considerably according to pulse times (Matthew *et al.*, 1988). Separate gels were run with pulse times of 25 s at 130 V for 18 h and 3 min at 130 V for 16 h. Compared to the linear size markers used (yeast chromosomes and lambda concatamers), the relative migration of the two extrachromosomal elements of *S. avermitilis* did not change as a result of variation of pulse times. Therefore, it is concluded that the extrachromosomal elements of *S. avermitilis* are linear plasmids. Both linear plasmids were observed to be present in *S. avermitilis* 31272.

In some species of *Streptomyces* it has been observed that certain plasmids can exist in more than one extrachromosomal form as a result of DNA rearrangements such as deletions and amplifications (Kieser *et al.*, 1982; Kinashi, 1989). To investigate if the two *S. avermitilis* plasmids were related, their ability to cross-hybridize was tested. Preparative PFGE was used to isolate the 250 kb

plasmid, which was subsequently purified and radio-labelled. The probe was hybridized to *S. avermitilis* DNA resolved by PFGE. It hybridized to the 250 kb plasmid but not to the smaller 100 kb plasmid, indicating that the two elements are unrelated. Hence, we conclude that *S. avermitilis* possesses two genetically dissimilar linear plasmids: one estimated at 100 kb, here designated as pSA1, the other of 250 kb, designated pSA2.

The pSA2 probe was also hybridized to DNA from *S. avermitilis* strain 31272 sc2 that no longer contains the 250 kb plasmid as visualized by PFGE. There was no hybridization to either extrachromosomal or chromosomal DNA, indicating loss of pSA2 without concomitant chromosomal integration. As sporulation and avermectin production are both normal in this strain, it can be concluded that pSA2 does not encode functions which have significant effect on these processes.

To investigate other functions that might be present on the plasmids, we hybridized a DNA fragment previously shown to be involved in pigment production in *S. avermitilis* (Tibbetts *et al.*, 1992). No hybridization was detected to either pSA1 or pSA2, although the probe did hybridize to chromosomal DNA. Hence, we conclude that pigment production is also not plasmid encoded. We are at present investigating other functions, including fertility, which could be encoded by one or other of the plasmids.

Site-specific DNA modification in *S. avermitilis*

The extensive degradation of *S. avermitilis* DNA in gels run in 0.5×TBE contrasted with samples run either in 1×HEPES or in 0.5×TBE supplemented with 50 µM thiourea (results not shown), indicating that fragmentation was not due to nuclease activity during insert preparation [as is evident for some other prokaryotes (Bancroft *et al.*, 1989)], but to modifications in the DNA which are susceptible to Tris-dependent strand scission as is the case for *S. lividans*. Because *S. lividans* DNA modifications are highly site-specific (Dyson *et al.*, 1986; Zhou *et al.*, 1988), we were interested in assessing the specificity of the *S. avermitilis* modifications.

Plasmid pIJ303 (Kieser *et al.*, 1982) was introduced into *S. avermitilis* 12804 and *S. lividans* and then re-isolated from both species. The DNA was then digested with either *Bam*HI, *Bgl*II, *Bsc*I, *Sac*I, *Xho*I (enzymes with unique recognition sequences in the plasmid) or combinations of any two of these enzymes, prior to induction of Tris-dependent site-specific cleavage. No fragmentation of pIJ303 DNA isolated from either *S. lividans* or *S. avermitilis* was detected after incubation with non-activated Tris. In contrast, incubation with activated Tris resulted in fragmentation of the plasmid isolated from both species, giving, in both cases, similar fragment profiles (Fig. 2a). The sizes of fragments produced by Tris-dependent cleavage were calculated for each single and double restriction enzyme digest of pIJ303, and a map of the 14 predominant cleavage sites was assembled (Fig. 2b). Cleavage did not result in equal proportions of every fragment, and the relative yield of each product was

Fig. 2. Site-specific Tris-dependent cleavage of pIJ303. (a) Plasmid DNA from *S. lividans* and *S. avermitilis* 12804 was linearized with *Bam*HI and incubated with either non-activated (lanes 1) or activated (lanes 2) 1×TAE buffer and then run on an agarose gel in 1×HEPES buffer as described. The DNA was transferred to a nylon membrane and hybridized with a digoxigenin-labelled pIJ303 probe. The sizes (kb) of photodigoxigenin-labelled bacteriophage lambda *Hind*III fragments (Boehringer Mannheim; lane 5) are indicated. (b) The locations of 14 Tris-dependent cleavage sites are indicated on the combined physical and genetic map of pIJ303, itself compiled from the map of the progenitor pIJ101 presented by Stein *et al.* (1989) and from data on the construction of pIJ303 by Kieser *et al.* (1982). The qualitative frequency of cleavage (high, intermediate or low) at individual sites is represented by triple, double and single arrowheads, respectively.

reproducible and similar for DNA isolated from both species. This may represent differences in the frequency of *in vivo* modification at different sites in both species, or it could be a consequence of variable affinity of the oxidative Tris derivative for modifications at different sites. By measuring peak sizes from densitometry traces of individual lanes, we assigned each site to one of three categories, representing high, intermediate or low yield of cleavage product. The location of only one of these preferential cleavage sites [site 14 in Fig. 2b has previously been mapped in the related plasmid pIJ101 (Zhou *et al.*, 1988)]. Investigation to allow comparison of the precise DNA sequence of different modification sites is currently underway.

Our results indicate that *S. avermitilis* possesses an unusual system of DNA modification, previously identified only in *S. lividans* (Dyson *et al.*, 1986; Zhou *et al.*, 1988). DNA modification in the two species is comparable both in terms of the chemical nature of the modifications, which

render them susceptible to Tris-dependent cleavage, and their site specificity. We are currently determining the nature and cause of this novel type of modification. In terms of DNA metabolism, these two species have previously been compared with regard to their ability to restrict methylated DNA (MacNeil, 1988). This study indicated that while *S. lividans* has a weak methyl-specific restriction system that can slightly reduce transformation frequencies with *N*⁶-methyladenine-containing DNA, *S. avermitilis* possesses a methyl-specific restriction system that severely reduces transformation frequencies with DNA containing either 5-methylcytosine or *N*⁶-methyladenine. Interestingly, it was observed that transformation of *S. avermitilis* with DNA from a methylase-deficient strain of *Escherichia coli* was less efficient than with DNA passed through *S. lividans*. It is possible that the novel DNA modifications introduced by *S. lividans* could confer on the DNA a higher efficiency of transforming *S. avermitilis*, which, as we have demonstrated, shares a similar modification system.

We are not aware of a systematic study into how widespread this type of DNA modification system is. That two well-studied but not obviously related species share the same phenomenon suggests it may occur in other actinomycetes. That *S. coelicolor* A3(2), a close relative of *S. lividans*, does not modify its DNA in this manner, suggests that not all species belonging to the same cluster group share the phenotype. The alternative conditions for PFGE we have used in this study should facilitate identification of other species which modify their DNA in a similar manner and enable physical analysis of their genomes.

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