Transformation of *Saccharopolyspora spinosa* protoplasts with plasmid DNA modified *in vitro* to avoid host restriction

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***Saccharopolyspora spinosa* protoplasts were not transformable by several different streptomycete plasmids, and *S. spinosa* was not a host for plaque formation by the *Saccharopolyspora* bacteriophages ϕ SE6, ϕ SE45, ϕ SE57, ϕ SE60, ϕ SE69 or HP10. Extracts of *S. spinosa* contained DNA-modifying activities that blocked cleavage of plasmid DNA by *NaeI* and *Sall*, and partially blocked cleavage by *NcoI*. Plasmid pOJ434, a derivative of the *S. spinosa* plasmid pSAS1, was modified *in vitro* by incubation with an extract of *S. spinosa* to circumvent restriction. Under optimal conditions, *S. spinosa* protoplasts were transformed to apramycin resistance by modified pOJ434 at frequencies of about 10^4 per μ g of DNA. Transformants contained pOJ434 primarily integrated in the chromosome.**

Keywords: *Saccharopolyspora spinosa*, protoplasts, transformation, bacteriophages

INTRODUCTION

Saccharopolyspora spinosa was isolated from soil in a defunct sugar-mill rum still in the Virgin Islands (Mertz & Yao, 1990). *S. spinosa* produces a novel insecticidal macrolide, A83543 (Kirst *et al.*, 1991). We are interested in exploring the structural organization and regulation of A83543 biosynthetic genes, and are developing methods to introduce DNA into *S. spinosa*. *S. spinosa* appears to express restriction endonucleases that block transformation by plasmid DNA and block plaque formation by *Saccharopolyspora* bacteriophages. In this report, we describe the development of a protoplast transformation system that uses host DNA modification activity in cell extracts to circumvent restriction by *in vitro* modification of plasmid DNA.

METHODS

Strains, plasmids and bacteriophages. *S. spinosa* ATCC 49460 (Mertz & Yao, 1990) was the host for some transformation and bacteriophage plaque-formation studies. *S. spinosa* PM89 is a novobiocin-treated derivative of ATCC 49460 lacking the plasmid pSAS1 (P. Matsushima & R. H. Baltz, unpublished). *Saccharopolyspora hirsuta* A70162 (NRRL 12045), *Saccharopolyspora* sp. A51741 and *Saccharopolyspora* sp. A51735 (Stanzak *et al.*, 1990) were hosts for preparing bacteriophage lysates as described by Cox & Baltz (1984). *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used for the preparation and isolation of plasmids bifunctional for *E. coli* and actinomycetes. *Streptomyces griseofuscus* (Cox & Baltz, 1984) was used to propagate plasmid pIJ702. The *Saccharopolyspora* bacteriophages

used were ϕ SE6, ϕ SE45, ϕ SE57, ϕ SE60, ϕ SE69 (Grund & Hutchinson, 1987) and HP10 (P. Matsushima & R. H. Baltz, unpublished). The plasmids used in transformation and DNA modification studies were pIJ702 (Katz *et al.*, 1983), pKC505 (Richardson *et al.*, 1987), pOJ160 (cited in Matsushima & Baltz, 1989), pKC796 (Kuhstoss *et al.*, 1991), pIJ4210 (Lydiat *et al.*, 1987), pCZA168 (Solenberg & Baltz, 1991) and pOJ434, a 6.5 kb vector derived from the *S. spinosa* plasmid pSAS1 that contains pUC12 for replication in *E. coli* and an apramycin resistance gene (Am^R) for selection in actinomycetes and *E. coli* (M. Bierman & B. Schoner, unpublished).

Media and culture conditions. *E. coli* DH5 α was grown in TY broth (Rao *et al.*, 1987) or TY broth plus 100 μ g Am ml⁻¹ (obtained from K. Merkel, Eli Lilly) to maintain plasmid DNA. The actinomycete strains were grown in TS broth and fragmented by ultrasonic vibration (Baltz, 1978). Protoplast regeneration was carried out on modified R2 medium (Baltz, 1978), modified R2M medium (Matsushima & Baltz, 1988), R2LE medium (Matsushima & Baltz, 1989), R2T20 medium (Yamamoto *et al.*, 1986) or R6 medium (Illing *et al.*, 1989). *S. spinosa* was grown in TS broth (Baltz, 1978) to prepare cell-free extracts for DNA modification experiments. TS agar (Baltz & Stonesifer, 1985) or R2LE agar supplemented with different antibiotics was used to determine the levels of antibiotic resistance expressed by *S. spinosa*. Bacteriophages were grown and titrated on *Saccharopolyspora* spp. or *Actinoplanes missouriensis* mycelial fragments on NC agar (Cox & Baltz, 1984). P medium was as described by Hopwood *et al.* (1985).

Protoplast formation and transformation. Protoplasts of *S. spinosa* were prepared by growing cells in TS broth plus 0.3% glycine or in R6 broth plus 0.3% glycine for different times, then incubating cells in P medium with 10 mg lysozyme ml⁻¹ for ~ 4 h at 23 °C (Baltz, 1978). The frequency of non-protoplasted

cells was determined by plating dilutions of the protoplast preparations on TS agar (Matsushima & Baltz, 1985). Typical protoplast preparations contained non-protoplasted cells at frequencies of 10^{-7} – 10^{-6} . The transformation procedures evaluated included those developed for *Streptomyces* spp. (Hopwood *et al.*, 1985; Matsushima & Baltz, 1985), *Amycolatopsis orientalis* (Matsushima *et al.*, 1987b), *Micromonospora rosaria* (Matsushima & Baltz, 1988) or *Saccharopolyspora erythraea* (Yamamoto *et al.*, 1986). The final procedure for transformation of *S. spinosa* PM89 by pOJ434 was as follows. Cells were grown in R6 broth plus 0.3% glycine for 48 h to an OD_{600} of about 2. Cells were washed and resuspended in P medium, then converted to protoplasts as described above. One microlitre 2 M NaCl was mixed with 6 μ l P medium and 1 μ l DNA. Undiluted protoplasts in P medium (200 μ l) and 30 or 40% PEG 6000 in P medium (500 μ l) were added to the DNA solution and mixed. The mixture was diluted and added in 4 ml of modified R2 soft agar (Baltz, 1978) to bottom agar containing about 30 ml R6 medium, dehydrated as described (Matsushima & Baltz, 1985). After 16–24 h incubation at 29 °C, the regenerating protoplasts were overlaid with 4 ml modified R2 soft agar containing Am to give a final concentration of 50 μ g ml $^{-1}$. The plates were further incubated for 7–10 d, then Am^R colonies were counted. Transformants were confirmed by Southern hybridizations with pOJ434.

Molecular techniques. General methods for manipulations of DNA were as described by Hopwood *et al.* (1985) and Sambrook *et al.* (1989). Southern hybridizations were carried out as described by Solenberg & Burgett (1989). Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Labs, Boehringer Mannheim Biochemicals, Amersham, United States Biochemical or Promega, and were used according to the specifications of the manufacturers.

In vitro modification of DNA. *S. spinosa* was grown in TS broth plus 0.3% glycine or R6 broth plus 0.3% glycine for 4–72 h (Baltz, 1978). Cells from 450 ml culture were washed in chilled PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl and 0.2 mM PMSF, pH 7.0), then resuspended in the same buffer plus 10 mg lysozyme ml $^{-1}$ to a final volume of ~10 ml. The cells were chilled in an ice bath for 10 min, then passed through a chilled French press [1 in (25.4 mm) piston; 20000 p.s.i. (138 MPa) at high ratio]; the extract was collected in a chilled tube. Cell debris was pelleted by centrifugation in the cold. The extract was decanted and each 3 ml of extract was mixed with 3 ml glycerol and 0.6 ml BSA (1 mg ml $^{-1}$), then stored at –20 °C.

The DNA modification assay included the following: 50–100 μ l TNE buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA]; 80 μ M S-adenosylmethionine; 100 μ g BSA ml $^{-1}$; 25 μ l extract; and 0.5–1.0 μ l DNA (L. Szynter & J. Brooks, personal communication). The mixture was incubated at 30 °C for 2–16 h, then extracted with phenol. The DNA was precipitated with ethanol, resuspended in TE buffer, and stored at –20 °C.

RESULTS AND DISCUSSION

Protoplast formation and regeneration

A prerequisite for transformation of protoplasts was to identify conditions for efficient formation of protoplasts and regeneration of cells from protoplasts. *S. spinosa* mycelia, grown for 48 h in TS broth plus 0.3% glycine or R6 broth plus 0.3% glycine, were readily converted to protoplasts in P medium after incubation with 10 mg lysozyme ml $^{-1}$ for about 4 h at 23 °C. Protoplast re-

generation was tested on several different media developed for regeneration of cells from protoplasts of *Streptomyces*, *Amycolatopsis*, *Micromonospora* or *Saccharopolyspora* spp. *S. spinosa* protoplasts regenerated poorly on modified R2, R2LE and R2M (i.e. 1–6% regeneration), and did not regenerate on R2T20 (< 10^{-6} per protoplast), a medium developed for *S. erythraea* plasmid transformation (Yamamoto *et al.*, 1986). *S. spinosa* protoplasts regenerated efficiently (~70%) on R6, a medium developed for *Streptomyces clavuligerus* protoplast transformation (Illing *et al.*, 1989).

Antibiotic resistance expressed in *S. spinosa*

S. spinosa mycelial fragments formed colonies at high efficiency (e.o.p. > 0.1) on 100 μ g spectinomycin ml $^{-1}$, 200 μ g hygromycin B ml $^{-1}$ and 50 μ g neomycin ml $^{-1}$. *S. spinosa* formed colonies at an e.o.p. of < 10^{-7} on R2LE medium plus 25 μ g Am ml $^{-1}$, 50 μ g streptomycin ml $^{-1}$ or 1 μ g thiostrepton ml $^{-1}$. *S. spinosa* cells grown in TS broth plus 0.1 μ g Am ml $^{-1}$ for 2 h plated at an e.o.p. of ~ 10^{-5} on R2LE plus 25 μ g Am ml $^{-1}$, suggesting that *S. spinosa* may express an inducible low-level resistance to Am. However, cells grown in the presence of 0.1 μ g Am ml $^{-1}$ formed colonies at efficiencies < 2×10^{-8} on R2LE plus 50 μ g Am ml $^{-1}$. Thus, 50 μ g Am ml $^{-1}$ was chosen to select transformants with plasmids encoding Am^R.

Attempts to demonstrate transformation with streptomycete plasmids

We attempted to transform *S. spinosa* protoplasts with pIJ702, pOJ160, and pKC505, using methods described for *Streptomyces* or *Saccharopolyspora* transformations (Hopwood *et al.*, 1985; Matsushima & Baltz, 1985; Yamamoto *et al.*, 1986), with R6 as the regeneration medium. Many parameters known to influence protoplast transformation frequencies were varied, including: the cell growth temperature; the culture density; the temperature for protoplast formation; the temperature for cell regeneration; the concentrations of DNA and protoplasts; and the source, M_r and concentration of PEG used to induce DNA uptake. None of the conditions tested yielded Am^R or Ts^R transformants. The lack of transformation suggested that the plasmids did not replicate, the resistance genes did not express, the plasmids were restricted, or a combination of problems existed.

Attempts to demonstrate plaque formation with *Saccharopolyspora* bacteriophages

In streptomycetes, the expression of restriction is reflected in the blockage of plaque-formation by several different bacteriophages (Cox & Baltz, 1984; Matsushima & Baltz, 1989). Non-restricting streptomycetes are permissive for plaque-formation by many broad-host-range streptomycete bacteriophages (Cox & Baltz, 1984; Matsushima *et al.*, 1987). To further probe if *S. spinosa* expressed restriction, we attempted to demonstrate plaque-formation by several different *Saccharopolyspora* bacteriophages. ϕ SE6, ϕ SE45, ϕ SE57, ϕ SE60 and ϕ SE69 lysates prepared on *S. erythraea* did not form plaques on *S. spinosa*.

All but ϕ SE60 were shown previously to form plaques on different *S. erythraea* strains and on *S. hirsuta* NRRL B-5792, the type strain for the genus *Saccharopolyspora* (Grund & Hutchinson, 1987). ϕ SE6 and ϕ SE69 also formed plaques on four other erythromycin-producing strains related to *S. erythraea* (Stanzak *et al.*, 1990). Since these bacteriophages formed plaques on several different *Saccharopolyspora* strains, the failure to form plaques on *S. spinosa* may be due to restriction.

We recently isolated a temperate bacteriophage from *S. hirsuta* A58143 (ATCC 20501) designated as HP10. HP10 formed plaques on *S. hirsuta* A55301 (ATCC 27875), *S. hirsuta* A70162 (NRRL 12045), *S. hirsuta* A58143, *S. erythraea* C233 (NRRL 2338), *Saccharopolyspora* spp. strains A37609, A51735, A51740, A51741, A51742, A51744 and A57583, and on *Actinoplanes missouriensis* A4696.8 (P. Matsushima & R. H. Baltz, unpublished). Thus, HP10 appeared to have a broad host specificity for *Saccharopolyspora* species. We prepared HP10 lysates on four *Saccharopolyspora* strains that appeared to express different restriction and modification of HP10, including *S. erythraea* C233, *S. hirsuta* A70162 and *Saccharopolyspora* spp. strains A51741 and A51735 (P. Matsushima & R. H. Baltz, unpublished), but none of the lysates was able to form plaques on *S. spinosa*. However, each phage preparation lysed *S. spinosa* at high multiplicities of infection. Furthermore, an HP10 lysate prepared on *S. erythraea* was shown to attach to *S. spinosa* in NC broth (Cox & Baltz, 1984) based upon a 1000-fold reduction in plaque-forming units after 6 h incubation at 32 °C. The combined results suggest that HP10 can attach to *S. spinosa* and cause lysis-from-without. We have also screened over 100 soil and fodder samples by enrichment procedures used to isolate streptomycete phages (Cox & Baltz, 1984); none have yielded bacteriophages that form plaques on *S. spinosa*. The combined bacteriophage enrichment and host range studies suggest that *S. spinosa* may express potent restriction systems that block bacteriophage replication.

Evidence for DNA modification in *S. spinosa*

Total DNA isolated from *S. spinosa* was incubated with different restriction endonucleases and analysed by agarose gel electrophoresis. *S. spinosa* DNA was cleaved by the following enzymes: *RsaI*, *HaeIII*, *HbaI*, *HpaII*, *AluI*, *Sau3AI*, *MboI*, *BstYI*, *MflI*, *ApaLI*, *PstI*, *BclI*, *PvuII*, *NlaIII*, *XhoII*, *SacI*, *SacII*, *XhoI*, *SphI*, *StuI*, *NruI* and *ScaI*. *S. spinosa* DNA was not cleaved by *NaeI*, *NcoI*, *SalI* or *DpnI*. The lack of cleavage by *DpnI* was consistent with cleavage by *MboI* and *Sau3AI*, indicating that *S. spinosa* does not express *MboI* restriction/modification. The lack of cleavage by the other three enzymes suggested that *S. spinosa* DNA may be modified at these cleavage sites. This is supported by the observation that *S. spinosa* DNA cloned in *E. coli* was cleavable by *NaeI*, *NcoI* and *SalI* (R. N. Rao, M. Bierman, M. Basinski & B. Schoner, personal communication). Extracts of *S. spinosa* also contain restriction endonuclease activity that cleaves *NcoI* sites (J. Brooks, personal communication), confirming that *S. spinosa* expresses isoschizomers for *NcoI* restriction and modification. The modification data for *SalI* and *NaeI*

restriction also suggest that corresponding isoschizomers for *SalI* and *NaeI* restriction endonucleases may be expressed in *S. spinosa*.

We isolated DNA from purified HP10 phage particles prepared on the four *Saccharopolyspora* strains discussed above (i.e. *S. erythraea* C233, *S. hirsuta* A70162 and *Saccharopolyspora* spp. strains A51741 and A51735) and analysed the DNA for cleavage by *NaeI*, *NcoI* and *SalI*. The DNA from all four strains was cleaved by *SalI* and *NcoI*. HP10 DNAs from lysates prepared on A70162, A51735 and A51741 were not cleaved by *NaeI*, but HP10 from C233 was partially cleaved by *NaeI* (P. Matsushima & R. H. Baltz, unpublished). These results indicate that *NaeI* modification, and presumably restriction, may be common in *Saccharopolyspora* spp., but that modification of *NaeI* sites in HP10 was not sufficient to allow HP10 to form plaques on *S. spinosa*. The lack of modification of *SalI* and *NcoI* sites in HP10 DNA by any of the *Saccharopolyspora* strains was consistent with the notion that *S. spinosa* may express *SalI*, *NcoI*, and possibly other restriction systems at levels that effectively block HP10 plaque formation.

Transformation of *S. spinosa* with pOJ434 modified *in vitro*

S. spinosa contains a plasmid pSAS1 that exists in both integrated and autonomous forms (M. Howell & R. N. Rao, unpublished). The native pSAS1 plasmid isolated from *S. spinosa* was shown to transform protoplasts of a *S. spinosa* strain cured of pSAS1, as measured by pock formation (Hopwood *et al.*, 1985) and Southern hybridization (M. Howell & R. N. Rao, unpublished), using R6 medium for optimum cell regeneration as described above. pOJ434, a derivative of pSAS1 (see Methods), contains two sites for *NaeI*, but no sites for *NcoI* or *SalI*. We attempted to transform *S. spinosa* protoplasts with pOJ434 prepared from *E. coli* without success. In an attempt to circumvent the apparent restriction barrier in *S. spinosa*, we prepared extracts of *S. spinosa* containing DNA-modifying activities, and incubated the extracts with pOJ434 prior to introducing the DNA into protoplasts. The DNA modification experiments were carried out under conditions designed to minimize restriction endonuclease activity (in a buffer containing EDTA and no Mg^{2+} ; see Methods). *S. spinosa* was grown in TS broth plus 0.3% glycine for 4–72 h and extracts were assayed for DNA-modification activities using pIJ702 (five *SalI*, four *NaeI* and two *NcoI* sites), pKC796 (three *SalI*, and no *NaeI* or *NcoI* sites), pIJ4210 (four *SalI*, six or seven *NaeI* and two *NcoI* sites), and pCZA168 (two *SalI*, three *NaeI* and one *NcoI* sites). [The numbers of sites were estimated by agarose gel electrophoresis (0.8%); small bands (< 0.5 kb) may not have been detected.] Extracts prepared from 24 or 48 h cultures gave good protection against cleavage by *SalI* (~75–100%); extracts from 24, 48 or 72 h cultures gave fair protection (~50–75%) for *NaeI*; and extracts from 4, 6 or 8 h cultures gave the best protection for *NcoI* (~50%). No modification was observed in controls lacking the methyl donor *S*-adenosylmethionine, but substantial cleavage of HP10

was observed with extracts containing 10 mM Mg^{2+} and no EDTA.

To maximize the modification of pOJ434 for *NaeI*, and possibly other restriction systems, we mixed *S. spinosa* extracts from 4 and 24 h cultures, and incubated the mixture with pOJ434. The treated and untreated pOJ434 preparations (2.5 µg each) were mixed with protoplasts prepared from *S. spinosa* PM89 (lacking pSAS1) grown for 48 h in R6 plus 0.3% glycine medium, and the mixture was treated with 30% PEG 6000. The protoplasts were plated on R6 medium and incubated at 29 °C for 16 h, then Am was added in modified R2 soft-agar overlays to give a final concentration of 50 µg ml⁻¹. The untreated pOJ434 gave < 0.4 transformants per µg of DNA, whereas the pOJ434 treated with the *S. spinosa* mixed extracts yielded about 400 transformants per µg of DNA. The presence of pOJ434 in putative transformants was verified by Southern hybridizations: pOJ434 was inserted into the *S. spinosa* chromosome at a unique site based upon the DNA fragment size profiles after cleavage of DNA with *Bam*HI and *Eco*RI. However, some free plasmid was also apparent (less than one copy per chromosome). Plasmid DNA prepared from one transformant transformed *S. spinosa* PM89 at a frequency of about 600 transformants per µg of DNA. These experiments indicated that pOJ434 can be modified to block *S. spinosa* restriction, and that *in vitro* modification is nearly as efficient as *in vivo* modification.

We have attempted to further improve the transformation frequency with modified pOJ434 by varying several parameters known to be important for streptomycete transformations. Growth of cells in R6 medium plus 0.3% glycine for 48 h to an OD₆₀₀ of ~2 before protoplast formation was optimal; growth for 24 h (OD₆₀₀ ~ 0.3) or 72 h (OD₆₀₀ ~ 4) yielded 10³-fold or twofold fewer transformants, respectively. The use of undiluted or 10-fold concentrated protoplasts gave similar results. PEG 6000 was superior to PEG 1000, and treatment of protoplasts and DNA with 30 or 40% PEG 6000 gave the best results; 10 and 20% PEG 6000 gave 100-fold and twofold fewer transformants, respectively. Addition of 0.8 µg calf thymus DNA (Matsushima & Baltz, 1985) had no effect, whereas addition of calf thymus DNA plus 1.5 µg protamine sulfate (Matsushima & Baltz, 1985) was detrimental. Addition of 0.25 M NaCl or 0.3 M sodium acetate (Matsushima & Baltz, 1985) gave a 50% increase in transformants. After incorporation of these minor adjustments in the transformation assay, modified pOJ434 yielded 4 × 10³ to 1 × 10⁴ transformants per µg DNA consistently. The frequency of transformants was proportional to DNA concentration between 0.5 and 2.5 µg DNA ml⁻¹.

Concluding remarks

We have developed gene transfer systems for a variety of actinomycetes (Matsushima & Baltz, 1985, 1988, 1989; Matsushima *et al.*, 1987b; McHenney & Baltz, 1988, 1989). *S. spinosa* has been the most recalcitrant strain for protoplast transformation that we have encountered. The

data presented here suggest that *S. spinosa* expresses an unusually effective set of restriction systems that protect it from bacteriophages and other foreign DNA. The breakage of the restriction barrier(s) by using the host's DNA-modification enzymes has proven to be a practical solution for the introduction of pOJ434 into *S. spinosa*. This may be a useful approach to help open other recalcitrant microbes to molecular genetic manipulations. The recent preliminary observation of conjugal transfer of plasmid DNA from *E. coli* into *S. spinosa* (Bierman *et al.*, 1992) is significant in light of the results presented here.

ACKNOWLEDGEMENTS

We thank M. Bierman, B. Schoner, D. Hopwood, C. R. Hutchinson and R. N. Rao for providing plasmids or bacteriophages; M. Bierman, J. Brooks, M. Howell, B. Schoner and R. N. Rao for communicating results prior to publication; L. Szynter and J. Brooks for providing advice on the DNA modification procedure; B. Fogleman for typing the manuscript; and DowElanco Company and Eli Lilly and Company for supporting this work.

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Received 14 June 1993; revised 23 August 1993; accepted 31 August 1993.