SHORT COMMUNICATION

Inhibition of Restriction in *Streptomyces clavuligerus* by Heat Treatment

By CHRISTOPHER R. BAILEY*† AND DAVID J. WINSTANLEY

*Beecham Pharmaceuticals, Research Division, Brockham Park, Betchworth,
Surrey RH3 7AJ, UK*

(Received 23 June 1986)

Inefficient transformation of *Streptomyces clavuligerus* protoplasts by DNA from the plasmid pIJ702, isolated from *S. lividans*, was attributed to restriction in view of the observation that efficient transformation was observed using modified pIJ702 (isolated from *S. clavuligerus*). The restriction system could be partially inhibited by treating protoplasts at 45 °C prior to transformation. This treatment increased the transformation frequencies of pIJ702 DNA by 100-fold and was used to introduce other plasmids into *S. clavuligerus*.

INTRODUCTION

Genes involved in antibiotic biosynthesis have been cloned from several *Streptomyces* spp. (Hopwood *et al.*, 1983; Martin & Gil, 1984; Malpartida & Hopwood, 1984; Bailey *et al.*, 1984; Ohnuki *et al.*, 1985). The interspecific transfer of such genes can lead to the production of novel antibiotics (Hopwood *et al.*, 1985). However, a potential limitation of this approach may be the occurrence of restriction systems, which appear to be widespread in *Streptomyces* (Cox & Baltz, 1984). Host-range limitations of bacteriophages have correlated well with the presence of restriction systems (Chater & Carter, 1978, 1979; Chater & Wilde, 1980; Cox & Baltz, 1984). Certain streptomycetes appear to be restriction-free. In *S. griseofuscus*, for example, lack of restriction of phage development was accompanied by efficient plasmid transformation (Cox & Baltz, 1984; Larson & Hershberger, 1984). In two restricting species, selection of restriction-deficient mutants has been achieved (Chater & Wilde, 1980; Hunter & Friend, 1984).

In this communication we demonstrate the presence of a restriction and modification system in *S. clavuligerus*, a species which produces several β -lactam antibiotics, including the β -lactamase inhibitor clavulanic acid (Reading & Cole, 1977). We also show the partial inhibition of *in vivo* restriction activity using heat treatment.

METHODS

Bacterial strains. These were *Streptomyces clavuligerus* ATCC 27064 and *Streptomyces lividans* 66 (John Innes Institute stock no. 1326).

Media and buffers. The compositions of YEME, P medium and R2YE (protoplast regeneration medium for *S. lividans*) are given in Chater *et al.* (1982). R5, the protoplast regeneration medium for *S. clavuligerus*, is described by Bailey *et al.* (1984). TSB is tryptone soya broth (Oxoid).

Preparation and transformation of protoplasts. Techniques for *S. lividans* were as described by Chater *et al.* (1982). The following modifications to the procedure were made for *S. clavuligerus*. Spores were inoculated into 25 ml TSB, in a 250 ml flask, and grown for 48 h at 26 °C, then 1 ml was transferred to a further 250 ml flask (with a coiled spring to disperse mycelial clumps) containing 10 ml TSB supplemented with 0.5% (w/v) glycine and 15 ml YEME + 34% (w/v) sucrose + 5 mM-MgCl₂ + 0.5% glycine. After 24 h, mycelium was harvested and protoplasts prepared according to Chater *et al.* (1982). Transformations were carried out using 10⁹ protoplasts and 25% (w/v) polyethylene glycol 1000 in P medium. Protoplasts were allowed to regenerate on R5 medium. Transformants were

† Present address: Apcel Ltd, 545/546 Ipswich Road, Slough, Berkshire SL1 4EQ, UK.

Table 1. Comparison of transformation frequencies in *S. clavuligerus* and *S. lividans*

The experiment was repeated on six occasions. Although absolute transformation frequencies varied (up to 10-fold) the relative differences within each experiment were constant. Representative data are given.

pIJ702 isolated from	Recipient	Transformants per µg pIJ702
<i>S. lividans</i>	<i>S. lividans</i>	2.4×10^6
<i>S. lividans</i>	<i>S. clavuligerus</i>	2.3×10^2
<i>S. clavuligerus</i>	<i>S. lividans</i>	1.4×10^6
<i>S. clavuligerus</i>	<i>S. clavuligerus</i>	1.1×10^6

Table 2. Effect of protoplast pre-incubation temperature on transformation frequency

The experiment was repeated on four occasions. Although absolute transformation frequencies varied (up to 10-fold) the relative differences within each experiment were constant. Representative data are given.

pIJ702 isolated from	Recipient	Room temp.	42 °C	45 °C	48 °C	50 °C
Transformants per µg DNA						
<i>S. clavuligerus</i>	<i>S. clavuligerus</i>	4.2×10^5	2.8×10^5	1.5×10^5	1.5×10^2	3.0×10^1
<i>S. lividans</i>	<i>S. clavuligerus</i>	5.0×10^1	8.0×10^2	5.4×10^3	4.0×10^1	0
<i>S. lividans</i>	<i>S. lividans</i>	3.6×10^6	1.2×10^6	2.0×10^5	7.1×10^4	1.0×10^3
Protoplast regeneration (%)						
	<i>S. clavuligerus</i>	10	10	10	0.04	0.01
	<i>S. lividans</i>	45	20	5	2	1

selected by overlaying regeneration plates with 2.5 ml soft nutrient agar containing 50 µg thiostrepton ml⁻¹ and incubating for 48 h at 26 °C to identify resistant colonies.

Heat treatment of protoplasts. This was done at the stage just prior to transformation. Protoplasts were pelleted and suspended in the small volume of P medium remaining after the supernatant was poured off. The tube was then incubated in a water bath for 10 min at the temperatures given in Table 2. Transformation was then carried out as above.

Isolation of plasmid DNA. The methods described by Kieser (1984) and Chater *et al.* (1982) were used.

RESULTS AND DISCUSSION

The 5.6 kb multicopy plasmid vector, pIJ702 (Katz *et al.*, 1983), was used in these studies. Firstly, a comparison was made of transformation frequencies in *S. clavuligerus* and *S. lividans*, a species containing no defined restriction barriers (Chater & Bruton, 1983). The results in Table 1 show that using pIJ702 prepared from *S. lividans* the frequency of transformation in *S. clavuligerus* was very low (10^4 fewer transformants than *S. lividans*). However, when pIJ702 was isolated from *S. clavuligerus* and used in transformation, the frequencies were high in both species. The most likely interpretation of these results is that *S. clavuligerus* possesses a restriction system, which leads to very inefficient transformation with foreign DNA, and a modification system, which allows DNA to avoid this restriction barrier. For self-cloning experiments in *S. clavuligerus*, the vector DNA must be modified (Bailey *et al.*, 1984).

In order to use *S. clavuligerus* as a recipient for foreign DNA we attempted to inhibit the restriction system by heat treatment. Table 2 shows the effect of protoplast pre-incubation temperature on transformation frequencies. At 45 °C there was a 10^2 -fold increase in frequency of transformation with pIJ702 prepared from *S. lividans*. At higher temperatures the viability of the protoplasts was severely impaired. Further experiments showed that heat treatment times up to 30 min gave no further increase in transformation frequency.

As there was no stimulation of transformation using modified DNA in *S. clavuligerus*, or in *S.*

lividans, the heat treatment appeared to be affecting the ability of *S. clavuligerus* to restrict foreign pIJ702 DNA. The effect is not specific to pIJ702 DNA as the technique was also used to introduce into *S. clavuligerus* SCP2-derived low-copy-number vectors, such as pIJ913 (Lydiat *et al.*, 1985), and SLP1-derived vectors, such as pIJ41 (Thompson *et al.*, 1982). Under standard conditions, no transformants were found using pIJ913 or pIJ41, but following heat treatment up to 1.0×10^3 transformants per μg were obtained.

There is now much interest in the production of new hybrid antibiotics by the transfer of cloned antibiotic biosynthetic genes isolated from one antibiotic producer into a recipient strain. We have demonstrated that in *S. clavuligerus* a potential barrier (an active restriction system) to this process can be inactivated by a simple procedure. This may allow the use of the industrially important strain, *S. clavuligerus*, as a recipient for 'foreign' antibiotic biosynthetic genes.

The authors would like to thank Dr M. J. Bibb of the John Innes Institute for fruitful discussion prior to this work being undertaken.

REFERENCES

- BAILEY, C. R., BUTLER, M. J., NORMANSELL, I. D., ROWLANDS, R. T. & WINSTANLEY, D. J. (1984). Cloning a *Streptomyces clavuligerus* genetic locus involved in clavulanic acid biosynthesis. *Biotechnology* **2**, 808–811.
- CHATER, K. F. & BRUTON, C. J. (1983). Mutational cloning in *Streptomyces* and the isolation of antibiotic production genes. *Gene* **26**, 67–78.
- CHATER, K. F. & CARTER, A. T. (1978). Restriction of a bacteriophage in *Streptomyces albus* P (CMI 52766) by endonuclease *Sal*PI. *Journal of General Microbiology* **109**, 181–185.
- 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. & WILDE, L. C. (1980). *Streptomyces albus* G mutants defective in the *Sal*GI restriction-modification system. *Journal of General Microbiology* **116**, 323–334.
- 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.
- COX, K. L. & BALTZ, R. H. (1984). Restriction of bacteriophage plaque formation in *Streptomyces* spp. *Journal of Bacteriology* **159**, 499–504.
- HOPWOOD, D. A., BIBB, M. J., BRUTON, C. J., CHATER, K. F., FEITELSON, J. S. & GIL, J. A. (1983). Cloning *Streptomyces* genes for antibiotic production. *Trends in Biotechnology* **1**, 42–48.
- HOPWOOD, D. A., MALPARTIDA, F., KIESER, H. M., IKEDA, H., DUNCAN, J., FUJII, I., RUDD, B. A. M., FLOSS, H. G. & OMURA, S. (1985). Production of 'hybrid' antibiotics by genetic engineering. *Nature, London* **314**, 642–644.
- HUNTER, I. S. & FRIEND, E. J. (1984). 'Restriction-deficient' mutants of industrial *Streptomyces*. *Biochemical Society Transactions* **12**, 643–644.
- KATZ, E., THOMPSON, C. J. & HOPWOOD, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703–2714.
- KIESER, T. (1984). Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- LARSON, J. L. & HERSHBERGER, C. L. (1984). Shuttle vectors for cloning recombinant DNA in *Escherichia coli* and *Streptomyces griseofuscus* C581. *Journal of Bacteriology* **157**, 314–317.
- LYDIAT, D. J., MALPARTIDA, F. & HOPWOOD, D. A. (1985). The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. *Gene* **35**, 223–235.
- MALPARTIDA, F. & HOPWOOD, D. A. (1984). Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. *Nature, London* **309**, 462–464.
- MARTIN, J. F. & GIL, J. A. (1984). Cloning and expression of antibiotic production genes. *Biotechnology* **2**, 63–72.
- OHNUKI, T., IMANAKA, T. & AIBA, S. (1985). Self-cloning in *Streptomyces griseus* of an *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. *Journal of Bacteriology* **164**, 85–94.
- READING, C. & COLE, M. (1977). Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. *Antimicrobial Agents and Chemotherapy* **11**, 852–857.
- THOMPSON, C. J., KIESER, T., WARD, J. M. & HOPWOOD, D. A. (1982). Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. *Gene* **20**, 51–62.