SHORT COMMUNICATION

Unstable Genetic Elements Affecting Streptomycin Resistance in the Streptomycin-producing Organisms Streptomyces griseus NCIB 8506 and Streptomyces bikiniensis ISP 5235

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(Received 29 May 1980)

There have been many cases in Streptomyces where a phenotype has proved to be unstable. This has usually been taken to suggest plasmid involvement. In the case of streptomycin resistance in two strains, this instability is reversible. Moreover, its cyclic nature suggests that insertion by a transposable element is affecting expression of a gene or genes.

INTRODUCTION

Both Streptomyces griseus and Streptomyces bikiniensis are producers of the antibiotic streptomycin. As such it would be expected that they would be resistant to the antibiotic at least during the time it is being produced (Demain, 1974), and this is the case. It has also been shown that resistance to streptomycin is an unstable character in these species (Freeman & Hopwood, 1978; Shaw & Piwowarski, 1977). Likewise, study of chloramphenicol resistance in Streptomyces coelicolor A3(2) showed that it was controlled by an unstable genetic element (Freeman et al., 1977) not directly involved with the two known plasmids from that species, SCP1 (Kirby et al., 1975) and SCP2 (Bibb et al., 1977). The loss of chloramphenicol resistance was totally reversible and cyclic.

Genetic instability has been studied in other species of Streptomyces. In Streptomyces alboniger (Redshaw et al., 1979) and Streptomyces cattleya (Kirby & O'Reilly, 1979), a high-frequency loss of argininosuccinate synthetase and the loss of the ability to produce aerial mycelium (Bld− phenotype) were found. These changes seemed, at least in the latter case, to be reversible to some extent and to affect other genes too. Hypotheses involving the loss of plasmid-encoded functions were suggested for the unstable phenotypes in S. bikiniensis and S. alboniger. In the other cases the changes were reversible showing that there had not been an irreversible loss of genetic material. Specifically in the case of S. coelicolor the phenotype did not show chromosomal linkage; thus a type of unstable genetic element other than a plasmid was indicated. In Escherichia coli, transposons and insertion sequences are responsible for reversible, unstable phenotypes (Cohen, 1976; Nevers & Saedler, 1977) and there is a strong possibility that similar elements are involved in Streptomyces.

METHODS

Strains. Streptomyces griseus NCIB 8506, Streptomyces bikiniensis ISP 5235 and Streptomyces bikiniensis ISP 5581, kindly provided by Glaxo Research Ltd, were used.

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Media. Minimal Medium (MM) was as described by Hopwood (1967). M-3 agar contained, per litre, 24 g Difco malt extract, 5 g Oxoid yeast extract and 15 g Oxoid agar no. 1.

General methods. The general methods used were those described by Hopwood (1967). All incubations were carried out at 30 °C. The conditions for ultraviolet-irradiation were as described by Kirby (1978a).

The minimum inhibitory concentration (m.i.c.) of streptomycin for the various strains was measured by placing 0.005 ml of filtered spore suspension on plates containing various concentrations of antibiotic. The plates were allowed to dry on the bench overnight and then incubated for 2 d; the lowest concentration at which no growth was observed was taken as the m.i.c.

Streptomycin production was measured using the plug assay method of Kirby (1978b), with *E. coli* C600 and C600 SmR as assay organisms. The mean zone diameter around four plugs was determined.

The method of isolation of covalently closed circular (ccc) DNA was a modification of that of Bibb et al. (1977) as described by Kirby & Wotton (1979).

RESULTS

The minimum inhibitory concentrations of streptomycin for the parental strains were: *S. griseus* NCIB 8506, 96 µg ml⁻¹; *S. bikiniensis* ISP 5235, 95 µg ml⁻¹; *S. bikiniensis* ISP 5581, 30 µg ml⁻¹.

*Streptomyces griseus* NCIB 8506. The frequency of clones sensitive to streptomycin at 60 µg ml⁻¹ was determined by replication from M-3 to MM and MM + antibiotic of both untreated and u.v.-irradiated spores after 3 d incubation (Table 1). Six streptomycin-sensitive clones chosen at random were restreaked and purified and their m.i.c. values were determined. Bld⁻ colonies were also observed both among streptomycin-sensitive (SmS) and streptomycin-resistant (SmR) colonies. The reversion frequency of these SmS strains to SmR was determined by plating on medium containing 80 µg streptomycin ml⁻¹ (Table 2). Only one SmS strain was Bld⁻ and this remained Bld⁻ on reversion to SmR. Three strains were retested for the instability of the reverted SmR phenotype (Table 2) and gave similar frequencies to the parental strain. None of the selected SmS clones was auxotrophic. Neither were auxotrophs induced on reversion to SmR on M-3 containing 120 µg streptomycin ml⁻¹. No effect on streptomycin production was detected using a plug assay except in the case of the Bld⁻ clone and this could be a pleiotropic effect of the mutation causing loss of aerial mycelium production.

*Streptomyces bikiniensis* ISP 5235. The frequency of clones which were sensitive to 60 µg streptomycin ml⁻¹ was determined (Table 1) and six SmS clones were chosen at random for further study. Their m.i.c. values were determined and also their reversion frequency to resistance to 80 µg streptomycin ml⁻¹ (Table 2). From these data, three classes were identified and this classification was supported when further SmS clones were isolated and their m.i.c. values determined.

Class 1: m.i.c. = 55 µg ml⁻¹; Bld⁺
Class 2: m.i.c. = 40 µg ml⁻¹; Bld⁻
Class 3: m.i.c. = <10 µg ml⁻¹; Bld⁻

One SmR revertant from each strain was studied further for the instability of its SmR phenotype (Table 2). Classes 2 and 3 gave rise at low frequency to Bld⁻ SmR clones which in most cases reverted to Bld⁺ SmS at a relatively high frequency. However, the stability of the SmR phenotype was decreased relative to the parent strain. This was particularly apparent in BK612, the strain derived from BK600 with the lowest m.i.c. (<10 µg ml⁻¹). The Bld⁻ phenomenon is similar to that described by Pogell (1979), Redshaw et al. (1979) and Kirby & O'Reilly (1979) except that in those cases there was often an associated arginine auxotrophy.

To determine whether in this case there was any effect on other genes, for example those affecting amino acid metabolism, the selection of SmS clones was repeated on complex medium, M-3. None of the SmS clones isolated (Table 1) was auxotrophic although 4.4% were Bld⁻. However, when BK598, BK600 and BK601 were reverted to SmR on M-3
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Table 1. Loss of streptomycin resistance in Streptomyces griseus NCIB 8506 and Streptomyces bikiniensis ISP 5235

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Total no. of colonies</th>
<th>Survival (%)</th>
<th>SmS colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCIB 8506</td>
<td>None</td>
<td>1112</td>
<td>100</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>U.v. (3 min)</td>
<td>409</td>
<td>37</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>368</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>U.v. (3 min)</td>
<td>448</td>
<td>73</td>
<td>1.4</td>
</tr>
<tr>
<td>ISP 5235</td>
<td>None</td>
<td>437</td>
<td>100</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>U.v. (3 min)</td>
<td>321</td>
<td>73</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1170</td>
<td>100</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>U.v. (3 min)</td>
<td>1821</td>
<td>22.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* M.i.c. ≤ 60 µg ml⁻¹.

Table 2. Reversion of SmS in Streptomyces griseus NCIB 8506 and Streptomyces bikiniensis ISP 5235

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Streptomycin production: zone size* (mm)</th>
<th>M.i.c.‡ (µg ml⁻¹)</th>
<th>Sporulation</th>
<th>Frequency of SmR†</th>
<th>Frequency of SmS from new SmR (%)</th>
<th>Spontaneous</th>
<th>U.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR581</td>
<td>NCIB 8506</td>
<td>12</td>
<td>25</td>
<td>Bld⁺</td>
<td>1.6 x 10⁻⁶</td>
<td>1.3</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>GR582</td>
<td>NCIB 8506</td>
<td>12</td>
<td>25</td>
<td>Bld⁺</td>
<td>1.4 x 10⁻⁴</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GR626</td>
<td>NCIB 8506</td>
<td>12</td>
<td>25</td>
<td>Bld⁺</td>
<td>8.7 x 10⁻⁴</td>
<td>2.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GR629</td>
<td>NCIB 8506</td>
<td>12</td>
<td>25</td>
<td>Bld⁺</td>
<td>9.7 x 10⁻⁴</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GR631</td>
<td>NCIB 8506</td>
<td>NZ</td>
<td>25</td>
<td>Bld⁻</td>
<td>4.1 x 10⁻⁴</td>
<td>1.7⁻‡</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GR633</td>
<td>NCIB 8506</td>
<td>12</td>
<td>25</td>
<td>Bld⁺</td>
<td>3.1 x 10⁻⁴</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK597</td>
<td>ISP 5235</td>
<td>13</td>
<td>55</td>
<td>Bld⁺</td>
<td>5.6 x 10⁻⁴</td>
<td>1.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK599</td>
<td>ISP 5235</td>
<td>13</td>
<td>55</td>
<td>Bld⁺</td>
<td>1.8 x 10⁻⁴</td>
<td>&lt;0.05</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK601</td>
<td>ISP 5235</td>
<td>14</td>
<td>55</td>
<td>Bld⁺</td>
<td>3.9 x 10⁻⁴</td>
<td>0.8-3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK598</td>
<td>ISP 5235</td>
<td>13-5</td>
<td>40</td>
<td>Bld⁻</td>
<td>1.5 x 10⁻⁴</td>
<td>2.2⁻‡</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK602</td>
<td>ISP 5235</td>
<td>13</td>
<td>40</td>
<td>Bld⁻</td>
<td>6.4 x 10⁻⁴</td>
<td>4.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BK600</td>
<td>ISP 5235</td>
<td>NZ</td>
<td>&lt;10</td>
<td>Bld⁻</td>
<td>9.8 x 10⁻⁷</td>
<td>§</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

NZ, No zone; ND, not determined.

* Zone sizes of parental strains: S. griseus NCIB 8506, 12 mm; S. bikiniensis ISP 5235, 13-5 mm.
† M.i.c. ≥ 95 µg ml⁻¹.
‡ New SmS are Bld⁻ while rest are Bld⁺.
§ Sectors Bld⁺/SmS at very high frequency.

containing 120 µg streptomycin ml⁻¹, all the isolated clones were Bld⁻ and 3/80, 2/72 and 4/80, respectively, were auxotrophic. Of these, six were not identifiable using standard amino acid and vitamin pools, two were Arg⁻ and one Met⁻.

A comparison of the streptomycin production of the various SmS strains is also shown in Table 2; the wild-type zone size was 13-5 mm. Although there is some variation in streptomycin production by the SmS strains, this does not seem to be significant except in the case of BK600 where no streptomycin production was detected.

Streptomyces bikiniensis ISP 5581. An attempt to select clones of this strain which were sensitive to 25 µg streptomycin ml⁻¹ failed to show any detectable instability of the SmR phenotype either spontaneously (<0.1%) or after u.v.-irradiation (<0.15%). Neither was it possible to detect a spontaneous forward mutation (<6.8 x 10⁻⁴) to increased streptomycin resistance (80 µg ml⁻¹).

Three attempts were made to isolate ccc DNA from each of the parental strains, with known plasmid-containing strains as a parallel control. No satellite bands were detected nor
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were ccc or open circular (oc) DNA molecules detected using the electron microscope. The method used to isolate the plasmid ccc DNA gave good quantities of ccc DNA from S. coelicolor A3(2), S. parvulus, S. fradiae (three strains) and S. jumonjinensis and oc and ccc molecules were detectable by electron microscopy in S. olivaceus and S. lipmanii.

DISCUSSION

The SmR phenotype of S. griseus is unstable. It undergoes a cyclic change from SmS to SmR and back which could not be due to plasmid loss. No other phenotypic effect on other genes is observed, nor is there any physical evidence for a plasmid. Thus, streptomycin resistance in this organism would seem to resemble chloramphenicol resistance in S. coelicolor A3(2) (Freeman et al., 1977) although in S. griseus it is not known if there is chromosomal linkage or not.

The SmR phenotype of S. bikiniensis is superficially similar to S. griseus in that it is cyclically unstable. This again shows that plasmid loss is not involved and neither is there any physical evidence for a plasmid. This disagrees with the conclusions of Shaw & Piwowarski (1977), although the results agree closely with their initial findings. The m.i.c. values of the SmS strains and their relative streptomycin production levels are the same. However, the change from SmS back to SmR is accompanied by changes in aerial mycelium production and induces auxotrophy. This, together with the different levels of sensitivity to streptomycin and the concomitant loss of streptomycin production in the case of the very sensitive BK600, implies that there are interactions with other genes.

The best hypothesis to explain these results would involve the insertion/deletion of a transposable element of some kind. If the element carried the genes responsible for streptomycin resistance, the phenotypic changes could be explained by its relationship with promoters of other genes. An alternative model would involve the insertion/deletion of an insertion sequence-type element into an operon controlling, in the case of S. bikiniensis, antibiotic resistance, antibiotic production and causing pleiotropic effects on aerial mycelium production and genes causing auxotrophy. A further possibility is that we are observing the effect of a controlling element which does not carry any genes involved in streptomycin resistance, nor directly interacts with the streptomycin resistance gene. However, its position or orientation on the chromosome could cause it to switch on or off streptomycin resistance and the other genes, either by acting cis from a location close to these genes or trans via an effector molecule.

These results throw light on the instability observed in many Streptomyces species and suggest that direct plasmid involvement may be less common than originally thought. The results do not completely eliminate the possibility that the genes for antibiotic production and resistance are plasmid-encoded in these strains but show that if they are, then changes in the plasmid are responsible for loss of antibiotic production and resistance, rather than plasmid loss.

REFERENCES


