SHORT COMMUNICATION

Two Erythromycin-resistance Plasmids of Diverse Origin and Their Effect on Sporulation in *Bacillus subtilis*

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A *Bacillus subtilis* plasmid capable of producing phenotypic erythromycin resistance was compared with an Ery^r staphylococcal plasmid. The two plasmids did not interfere with the sporulation process in *B. subtilis*, in contrast to chromosomal erythromycin mutations.

INTRODUCTION

Analysis of sporulation and germination in *Bacillus subtilis* using recombinant DNA technology has been handicapped by the lack of *Bacillus* plasmids with selectable markers (Lovett & Bramucci, 1974; Tanaka *et al.*, 1977; Bernhard *et al.*, 1978). For this reason, soil samples were examined for the presence of bacilli which might contain plasmid molecules that could confer resistance to specific antibiotic compounds.

The isolation of a plasmid which determines resistance to the macrolide antibiotic, erythromycin, encouraged us to investigate the sporulation of host strains carrying either this erythromycin resistance (Ery^r) plasmid or an Ery^r staphylococcal plasmid capable of replicating in *B. subtilis* (Weisblum *et al.*, 1979). Strains of *B. subtilis* which are Ery^r due to chromosomal mutations all show drastic alteration of the normal sporulation pattern (Domoto *et al.*, 1975; Tipper *et al.*, 1977).

METHODS

Bacterial strains used are listed in Table 1.

Gram-positive, rod-shaped micro-organisms were isolated from local soil by plating diluted soil samples on nutrient agar (Difco) plates. The purified strains were then tested for antibiotic resistance by streaking on antibiotic medium 3 (Difco) plates containing either tetracycline, chloramphenicol, kanamycin or erythromycin.

Covalently closed circular DNA was isolated from *B. subtilis* RC242 (see Table 1) by the method of Tanaka & Sakaguchi (1978). Chromosomal DNA was isolated as described by Saito & Miura (1963).

For determining the plasmid copy number, cells were labelled with [*methyl*-³H]thymidine (New England Nuclear) by the method of Lovett & Bramucci (1974).

The buoyant density of plasmid DNA was determined by equilibrium centrifugation in CsCl performed in a Beckman model E analytical ultracentrifuge as described by Schildkraut *et al.* (1962). Restriction enzymes were purchased from Biolab (Beverly, Mass., U.S.A.).

For transformation experiments, cells were made competent by the procedure of Bott & Wilson (1967). Minicells were isolated and labelled as described by Shivakumar *et al.* (1979). For sporulation experiments, cells were grown to an absorbance (600 nm) of 0.5 to 0.6 in the medium described by Schaeffer *et al.* (1965) and resuspended to the same absorbance at room temperature in Sterlini & Mandelstam (1969) resuspension medium. The percentage of sporulation was determined by the ratio of the number of heat-resistant spores (10 min at 80 °C) counted at T24 (24 h after the end of exponential growth) to the number of vegetative cells present at T2 (2 h after the end of exponential growth).

Table 1. *Strains of Bacillus subtilis* used

Strain	Chromosomal markers	Sporulation phenotype	Resistance/sensitivity to erythromycin	Plasmid	Origin
RC242		Spo ⁺	r	pIM13	This laboratory
1A29	<i>met leu ade</i>	Spo ⁺	s		J. Copeland
RC248	<i>met leu ade</i>	Spo ⁺	r	pIM13	1A29
RC241†	<i>ade recE</i>	Spo ⁺	s		1A29
RC244	<i>ade recE</i>	Spo ⁺	r	pIM13	1A29
RC243	<i>met leu ade ery</i>	Spo ⁻	r		1A29
168‡	<i>ery</i>	Spo ⁻	r		This laboratory
CU403	<i>thyA thyB metB div 1V-B1</i>	Spo ⁺	s		D. Dubnau
RC245	<i>thyA thyB metB div 1V-B1</i>	Spo ⁺	r	pIM13	CU403
BD477	<i>thyA thyB metB div 1V-B1</i>	Spo ⁺	r	pBD15	B. Weisblum

† Transformant of 1A29 with DNA from BD224 *recE* (Dubnau *et al.*, 1973).

‡ Spontaneous mutant of *B. subtilis* 168.

RESULTS AND DISCUSSION

Among the antibiotic-resistant strains isolated from soil samples, one identified as *Bacillus subtilis* (Buchanan & Gibbons, 1974) proved resistant to 2 µg erythromycin ml⁻¹. This isolate, RC242 (Table 1), contained plasmid DNA (pIM13), a small 1.5 × 10⁶ daltons (2.2 kilobases) molecule (Fig. 1). Plasmid pIM13 DNA had the same buoyant density of 1.703 g ml⁻¹ as *B. subtilis* DNA (Schildkraut *et al.*, 1962) and contained a single site for the restriction enzymes *Hha*I and *Sac*I. Two sites were present for *Hind*III which produced a major (1.4 × 10⁶ daltons) and a minor (0.1 × 10⁶ daltons) fragment.

A copy number of approximately 15 was calculated from the amount (1.1 %) of ³H radioactivity present in the covalently closed circular band of caesium chloride-ethidium bromide gradients compared with the amount of ³H radioactivity in total DNA. The molecular weight of the *B. subtilis* chromosome was taken as 2 × 10⁹ daltons (Kavenoff, 1972).

When (recombinant) *rec*⁺ and *recE* strains of *B. subtilis* were transformed with pIM13 DNA, 5 × 10³ erythromycin-resistant transformant colonies ml⁻¹ were obtained. While the plasmid DNA transformed both *rec*⁺ and *recE* recipient cells, chromosomal DNA isolated from the original Ery^r soil isolate RC242 transformed neither. Chromosomal DNA obtained from a spontaneous Ery^r mutant of *B. subtilis* 168 produced 10⁵ erythromycin-resistant transformant colonies ml⁻¹ in the *rec*⁺ strain and no transformants in the *recE* strain.

Strain BD477 contained an Ery^r plasmid, pBD15, originally isolated from *Staphylococcus aureus* and transferred to strains of *B. subtilis* by transformation (Weisblum *et al.*, 1979). The staphylococcal plasmid DNA (2.3 × 10⁶ daltons) differed from pIM13 DNA in its buoyant density of 1.692 g ml⁻¹ and in possession of a single recognition site for the enzymes *Hae*III and *Xba*I (Weisblum *et al.*, 1979). Transformation of *rec*⁺ and *recE* strains produced 10⁵ erythromycin-resistant transformant colonies ml⁻¹.

Interference with the normal pattern of sporulation of Ery^r strains of *B. subtilis* has been described by several investigators (Domoto *et al.*, 1975; Graham & Bott, 1975; Tipper *et al.*, 1977). A large number of independently isolated chromosomal Ery^r mutants have been reported to display the following characteristics: normal sporulation at 30 to 35 °C, but less than 1 % sporulation at 47 °C, and the inability to form mature spores in the presence of erythromycin at either 30 or 47 °C (Goldman & Tipper, 1979). Vegetative growth of all Ery^r strains was not affected by the same concentration of erythromycin.

We wished to compare sporulation in strains which were resistant to erythromycin due to the presence of a plasmid-borne resistance factor with those in which the antibiotic resistance arose from chromosomal alterations. Two of our chromosomal Ery^r strains – 168 Ery^r, a

Fig. 1

Fig. 2

Fig. 1. Electrophoresis of pIM13 DNA in 0.7 % agarose (Seakem, Rockland, Maine 04841, U.S.A.). Bromophenol blue (0.25 %) in 70 % sucrose was added to the samples (10 to 25 µl) and the horizontal slab gel was run at 25 mA for approximately 10 h. The gel was stained in ethidium bromide solution (1 µg ml⁻¹) for 15 min and photographed under short-wavelength ultraviolet light. A yellow filter (Kodak no. 9 Wratten gelatin) and Polaroid 3000 speed, type 107 film were used. (a) Native pIM13 DNA, (b) digested with *Hha*I, (c) digested with *Sac*I, (d) digested with *Hind*III and (e) lambda phage digested with *Hind*III (standards, on the right, are expressed in megadaltons).

Fig. 2. Polypeptides specified by Ery^r plasmids. Minicells (100 µl) containing either pIM13 or pBD15 were incubated with [³⁵S]methionine [The Radiochemical Centre, Amersham; 1.05 Ci mmol⁻¹ to a final concentration of 5 µCi ml⁻¹ (185 kBq ml⁻¹)] for 30 min. Lysis of minicells was accomplished as described by Shivakumar *et al.* (1979) and the polypeptides synthesized were resolved on a sodium dodecyl sulphate-polyacrylamide gel, prepared as described by Studier (1973). To obtain autoradiograms, the dried gel was exposed to X-ray film (Kodak-XR2) at -70 °C. Lanes a to c contain polypeptides of pBD15 produced in the presence of (a) 1 µg erythromycin ml⁻¹, (b) 0.1 µg erythromycin ml⁻¹ and (c) no erythromycin. Lanes d and e contain polypeptides of pIM13 produced in the presence of (d) no erythromycin and (e) 1 µg erythromycin ml⁻¹.

Molecular weight markers used: phosphorylase A (92500 daltons), bovine serum albumin (67000 daltons), glutamate dehydrogenase (53000 daltons), glyceraldehyde-3-phosphate dehydrogenase (36000 daltons), carbonic anhydrase (30000 daltons).

spontaneous mutant, and RC243, which was transformed to antibiotic resistance – were examined. Both strains failed to sporulate normally at 47 °C or at 30 °C in the presence of 1.5 µg erythromycin ml⁻¹. In contrast, strain RC242, three strains transformed with pIM13 DNA and strain BD477 sporulated normally both at low and high temperature and in the presence of the antibiotic (Table 1).

Since the synthesis of proteins coded by pBD15 in minicells of *B. subtilis* had been reported (Shivakumar *et al.*, 1979), a comparison of the pattern of polypeptides coded by this staphylococcal plasmid with the *Bacillus* plasmid pIM13 was undertaken. Minicells were isolated from strains BD477 and RC245, labelled with [³⁵S]methionine and, after lysis (Shivakumar *et al.*, 1979), loaded on to a polyacrylamide–sodium dodecyl sulphate gel. As reported (Shivakumar *et al.*, 1979), no protein synthesis was observed in plasmid-free preparations of minicells.

Several polypeptide bands were common to both plasmids (Fig. 2). Of these, two bands – one of 45000 and one of 29000 daltons – produced by pBD15, a multi-copy mutant, were particularly intense. The 29000 dalton protein is erythromycin-inducible (Shivakumar *et al.*, 1979). Plasmid pIM13 also produced a faint band of 29000 and a stronger band of 45000 daltons. However, lane *e* in Fig. 2, which represents proteins from minicells labelled in the presence of erythromycin, shows the appearance of a new polypeptide of approximately 41000 daltons (arrow). Some changes in the antibiotic-inducible proteins of Ery^r plasmids from unrelated host sources is perhaps not too surprising.

The studies of Tipper *et al.* (1977) have revealed that the defect in all chromosomal Ery^r mutants examined to date results from an alteration of a structural gene for ribosomal protein L-17. They suggest that the modification produced in *B. subtilis* disturbs one or more sporulation-specific ribosomal functions. Plasmid-coded erythromycin resistance, as examined in strains of staphylococci and streptococci, results in the methylation of 23S ribosomal RNA (Weisblum, 1974). The ribosomal modification produced by a staphylococcal Ery^r plasmid does not seem to interfere with sporulation in *B. subtilis*. Normal spore morphogenesis also occurs in the presence of an Ery^r *Bacillus* plasmid whose properties have been described in this communication.

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