Susceptibility to Butirosin and Neomycin B in *Bacillus circulans*, the Butirosin-producing Organism

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Butirosin, an aminoglycoside antibiotic, is produced by *Bacillus circulans* B-3312. Experiments using recombined ribosomal and supernatant fractions from this strain and from *B. megaterium* KM have shown that the ribosomes of both are sensitive to butirosin. The aminoglycoside 3'-phosphotransferase present in *B. circulans* modifies butirosin and neomycin *in vitro* but confers resistance only to the former *in vivo*. The phosphotransferase does not modify a detectable amount of extracellular butirosin while mediating resistance to the antibiotic. *In vitro*, however, the enzyme appears to protect against inhibition by butirosin by inactivating the bulk of the antibiotic in the system. An extrachromosomal element of unknown function has been detected in *B. circulans*.

INTRODUCTION

The mechanisms by which antibiotic-producing organisms determine resistance to their own antibiotics are of considerable practical and theoretical interest (Demain, 1974). *Bacillus circulans* B-3312, the bacterium which produces butirosin, an aminoglycoside antibiotic related to neomycin, has been shown to synthesize an aminoglycoside 3'-phosphotransferase which will modify butirosin and other aminoglycosides *in vitro* (Dowding & Davies, 1975). The genetic determinant of this enzyme, which was assumed to be chromosomal, was transferred to *Escherichia coli* and shown to confer resistance to butirosin and a wider range of related compounds (Courvalin *et al.*, 1977).

A second butirosin-producing bacterium, *Bacillus vitellinus* Z-1159, has also been shown to synthesize an aminoglycoside 3'-phosphotransferase. On the basis of its substrate specificity, it was suggested that this enzyme, which appears to be very similar to the *B. circulans* enzyme, might be responsible for conferring butirosin resistance on *B. vitellinus* (Nakahama *et al.*, 1977).

This paper describes studies on the mechanism of resistance to butirosin and related compounds in *B. circulans*.

METHODS

Bacterial strains. *Bacillus circulans* B-3312 is one of the two original butirosin-producing isolates (Howells *et al.*, 1972) and was obtained from Dr T. G. Pridham, Northern Regional Research Laboratories, Peoria, Ill. 61604, U.S.A. *Bacillus megaterium* KM was obtained from Dr E. Cundliffe, Department of Biochemistry, University of Leicester.

Preparation and assay of crude extracts. Bacteria were grown and extracts were prepared as previously described (Dowding, 1977). Aminoglycoside-modifying enzymes and antibiotic concentrations were assayed by the phosphocellulose paper binding technique (Haas & Dowding, 1975).

Preparation of supernatant and ribosomal fractions. *Bacillus circulans* and *B. megaterium* were grown by diluting 10 ml of an overnight culture into 1 l ML medium (g l⁻¹: Difco Bacto-Tryptone, 10; Difco Bacto-yeast extract, 5; NaCl, 10). Flasks were incubated at 37 °C with shaking until an A_{550} of 1.0 to 1.4 was reached.

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The cells were harvested, washed once in buffer I (10 mM-Tris/acetate pH 7.8, 10 mM-magnesium acetate, 25 mM-ammonium acetate, 2 mM-dithiothreitol) and ground with twice their wet weight of alumina. Buffer I (5 ml) was then added and the crude homogenate was centrifuged at low speed to remove alumina and any unbroken cells. The extract was then centrifuged at 30000g for 30 min and the supernatant (S30), held in ice, was dialysed against two changes (2 l each) of buffer I. The ribosome concentration in the S30 fraction was normally 5 to 10 mg ml⁻¹. The S30 fraction was centrifuged at 150000g for 2.5 h and fractions of the supernatant (S150) were stored at -20 °C. The crude ribosome pellet was resuspended in 5 ml buffer I at 4 °C, centrifuged at low speed to remove debris and re-pelleted at 150000g for 2.5 h. The clear ribosome pellet was resuspended in buffer I (one tenth of the volume of the original S30) and fractions were stored at -20 °C.

Polyphenylalanine synthesis in vitro. Polyuridylic acid [poly(U)]-directed polyphenylalanine synthesis was assayed as follows. Each reaction mixture contained: 10 µl HKM buffer [200 mM-N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (BDH) pH 7.5, 500 mM-potassium acetate, 85 mM-magnesium acetate]; 10 µl of a solution containing 0.4 mM-amino acids (except phenylalanine), 25 mM-ATP (Boehringer), 4 mM-GTP (Boehringer), 50 mM-phosphoenolpyruvate (Boehringer) pH 7.2; 10 µl L-[U-¹⁴C]phenylalanine (The Radiochemical Centre, Amersham; 50 µCi ml⁻¹, approx. 500 mCi mmol⁻¹); 5 µl poly(U) (Boehringer; 10 mg ml⁻¹ in water); 5 µl transfer RNA (Sigma; *E. coli*, unfractionated; 20 mg ml⁻¹); 5 µl pyruvate kinase (Boehringer; 1 mg ml⁻¹ in glycerol); 5 µl water; 45 µl supernatant (S150); 5 µl ribosome suspension. Antibiotic (or water in control experiments) was added after 5 min incubation at 30 °C. At various times samples (10 µl) were removed to tubes containing 1 ml 10% (w/v) trichloroacetic acid (TCA). Samples were heated at 90 to 100 °C for 20 min to hydrolyse transfer RNA and filtered on to Whatman GF/C glass fibre discs. Filters were each washed with approximately 20 ml 5% (w/v) TCA, dried and counted using 0.4% (w/v) 2-(4'-*tert*-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (butyl-PBD) in toluene as scintillant.

Caesium chloride gradient centrifugation. An overnight culture (1 ml) of *B. circulans* was diluted into 20 ml ML medium containing 1% (w/v) glucose and incubated with shaking at 37 °C for 2.5 h; 2'-deoxyadenosine (Sigma) was added (final concentration 200 µg ml⁻¹) followed by [*methyl*-³H]thymidine (approx. 15 Ci mmol⁻¹; The Radiochemical Centre) to give 10 µCi ml⁻¹. Growth was continued for 3 h; the cells were then harvested, washed once in ML medium and lysed as described by Guerry *et al.* (1973). The crude extract was centrifuged at 30000g for 30 min, mixed with ethidium bromide (final concentration 250 µg ml⁻¹) and saturated CsCl solution was added to yield a density of 1.56 g ml⁻¹. Gradients were centrifuged at 42000 rev. min⁻¹ for 38 to 40 h in the Ti-75 rotor of a Beckman Spinco L5-65. Fractions were collected on Whatman 3MM paper discs which were then washed twice in 5% (w/v) TCA, twice in ethanol/ether (1:1, v/v) and once in ether. Discs were air-dried and counted in toluene containing 0.4% (w/v) butyl-PBD as scintillant.

RESULTS

Cell-free protein synthesis

Figure 1 shows the effect of butirosin on recombined systems from *B. circulans* and *B. megaterium*. *Bacillus megaterium* was used as a control in these experiments as it is not known to produce any antibiotic, it was found to contain none of the known aminoglycoside-modifying enzymes and, being a member of the same genus, it might be expected to yield active mixed cell-free systems with *B. circulans*. Ribosomes and supernatants from the two strains could be recombined to give four systems which actively synthesized polyphenylalanine in response to poly(U); controls in the absence of poly(U) or of ribosomes showed negligible synthesis. Ribosomes of *B. circulans* and *B. megaterium* were sensitive to butirosin when assayed in the *B. megaterium* S150 fraction but both could be protected by supernatant from the phosphotransferase-containing strain *B. circulans*. In the case of both types of ribosome with the *B. circulans* S150 fraction, there was a lag of approximately 2 to 3 min after antibiotic addition before synthesis was resumed at the previous rate. This is assumed to represent the time taken by the enzyme to lower the butirosin concentration to a sub-inhibitory level. This conclusion is supported by separate assays of the time course of butirosin phosphorylation *in vitro*; these assays also revealed the presence of an unspecified ATPase activity in extracts of *B. circulans*.

Fig. 1. Poly(U)-directed polyphenylalanine synthesis by recombined ribosomal and supernatant fractions from *B. circulans* and *B. megaterium* (●) and the effect of butirosin ($30 \mu\text{g ml}^{-1}$) on this synthesis (▲). The arrow indicates the time of addition of butirosin. (a) *B. circulans* ribosomes plus *B. megaterium* supernatant; (b) *B. circulans* ribosomes plus *B. circulans* supernatant; (c) *B. megaterium* ribosomes plus *B. megaterium* supernatant; (d) *B. megaterium* ribosomes plus *B. circulans* supernatant.

Resistance to aminoglycosides *in vivo*

The effect of two structurally related aminoglycosides (butirosin and neomycin B) on the growth of *B. circulans* was tested. The antibiotics were added to exponentially growing cultures in ML medium (doubling time 48 min) at an A_{550} of 0.2. Although neomycin B is a better substrate for the phosphotransferase than butirosin *in vitro* (Courvalin *et al.*, 1977; J. E. Dowding, unpublished results), growth of *B. circulans* was completely inhibited by neomycin B but was unaffected by butirosin. Measurement of the concentration of antibiotic remaining in the supernatant during the course of the two experiments showed that in neither case did the concentration fall measurably from the initial level ($30 \mu\text{g ml}^{-1}$). Thus resistance of *B. circulans* to butirosin is not mediated by inactivation of the bulk of the antibiotic in the medium.

Plasmid DNA in *B. circulans*

There has recently been increasing interest in the involvement of plasmids in both antibiotic production (Hopwood, 1978) and antibiotic resistance in antibiotic-producing organisms (Freeman & Hopwood, 1978). This prompted the examination of *B. circulans* for the presence of extrachromosomal DNA. The distribution of radioactive material in a typical buoyant density gradient demonstrates that a plasmid is present (Fig. 2). Attempts to transform *E. coli* C600 with this plasmid by the method of Cosloy & Oishi (1973) were unsuccessful. Plasmid DNA was isolated from CsCl gradients or prepared as described by Guerry *et al.* (1973) and selection was with a range of concentrations (10 to $50 \mu\text{g ml}^{-1}$) of

Fig. 2. Caesium chloride-ethidium bromide density gradient of *B. circulans* DNA labelled with [methyl- ^3H]thymidine.

butirosin or neomycin. A number of colonies resistant to both butirosin (up to $30\ \mu\text{g ml}^{-1}$) and neomycin (up to $20\ \mu\text{g ml}^{-1}$) were obtained with either selective agent, but none of those tested contained detectable butirosin-phosphorylating activity. These isolates were assumed to be low-level permeability mutants and were not examined for the presence of plasmid DNA. Although inconclusive, these experiments suggested that the plasmid does not code for the phosphotransferase; whether it is involved in butirosin synthesis in any way remains to be determined.

DISCUSSION

It is becoming clear that different antibiotic-producing organisms respond to the compounds they synthesize with fundamentally different mechanisms of self-defence. *Streptomyces azureus*, for example, which produces the ribosome-inhibitor thiostrepton, has recently been shown to methylate its ribosomal RNA and thereby render the target for the antibiotic refractory to it (Cundliffe, 1978). The cell-free protein synthesis experiments reported here show that the ribosomes of *B. circulans* are sensitive to butirosin, also an inhibitor of ribosomal function, although they can be protected by the supernatant fraction which contains phosphotransferase activity.

This paper confirms the earlier finding (Courvalin *et al.*, 1977) that although the enzyme can modify neomycin *in vitro* this is apparently insufficient to confer resistance *in vivo*. This contrasts with the situation in *E. coli* (Courvalin *et al.*, 1977) where the same enzyme has been reported to determine resistance to neomycin as well as to butirosin and other aminoglycosides. In these experiments, however, selection in *E. coli* was with neomycin and this technique may have selected a cell-enzyme combination with altered permeability to aminoglycosides and hence a different resistance phenotype.

As yet, there is only circumstantial evidence that the modifying enzyme present in *B. circulans* mediates resistance to butirosin in this organism, as opposed to being involved in an earlier stage of butirosin synthesis for example, although the experiments described here and elsewhere (Courvalin *et al.*, 1977; Nakahama *et al.*, 1977) make this seem very likely. Direct evidence would come from the isolation of a butirosin-sensitive mutant which had lost the ability to produce the phosphotransferase. As such a mutation may be lethal in the event of butirosin synthesis, this type of strain would have to be maintained under

conditions unfavourable for antibiotic production or, alternatively, isolated in a non-producing mutant of the parent organism. Any role in antibiotic production (or resistance) of the plasmid detected in *B. circulans* may, likewise, be elucidated by the isolation of 'cured' derivatives.

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