

AMINOGLYCOSIDE 3'-PHOSPHOTRANSFERASE IN *BACILLUS CIRCULANS* PRODUCING BUTIROSINS

Sir:

As reported in previous papers,^{1,2,3)} three types of aminoglycoside 3'-phosphotransferases [APH (3')-I, II and III] have been found in resistant strains of *Escherichia coli* carrying R factors and *Pseudomonas aeruginosa*. Moreover, as reported by DAVIES *et al.*^{4,5)} similar enzymes have also been found in *Streptomyces fradiae*, *Streptomyces ribosidificus* and *Bacillus circulans* which produce neomycins, ribostamycin and butirosins, respectively. However, the relationships of these enzymes in the strains producing the antibiotics

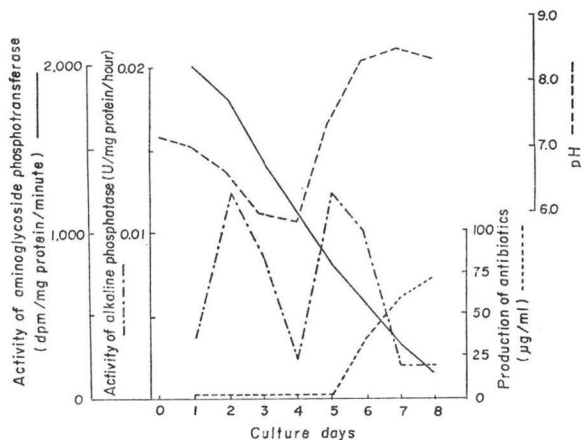
to those enzymes in resistant bacteria and the structures of the enzyme reaction products have not yet been completely studied. In this communication, we report that the enzyme in *Bacillus circulans* transfers the phosphate from ATP to the 3'-hydroxyl group of butirosin A, and that this enzyme can be distinguished from the enzymes in the resistant strains by the immunological behavior. We also report changes in activity of intracellular phosphotransferase and phosphatase during the growth of *Bacillus circulans* and the production of the butirosins.

A strain of *Bacillus circulans* (NRRL B-3313) was shake-cultured in a medium containing 4% glycerol, 1.75% Polypeptone, 1% soybean meal, 0.5% ammonium chloride, 0.4% calcium

Fig. 1. Activities of intracellular phosphotransferase and alkaline phosphatase, pH of the cultured broth and production of antibiotics as butirosin A during the shaking culture of *Bacillus circulans* NRRL B-3313.

The composition of the medium and the culture are described in the text. For determination of activities of phosphotransferase and phosphatase, the cells were harvested at the indicated times and the S-100 fractions were prepared as described in the text. The volumes of the cultured broths taken were as follows: 250 ml on day 1, 250 ml on day 2, 125 ml on day 3, 125 ml on day 4, 125 ml on day 5, 125 ml on day 6, 125 ml on day 7, 125 ml on day 8.

The phosphotransferase activity was determined as follows: the mixture (250 μ l) containing 0.2 mM butirosin A, 16 mM adenosine triphosphate, 0.5 μ Ci of γ -³²P-adenosine triphosphate (15.4 Ci/mmol, purchased from the Radiochemical Centre Ltd., Amersham), 10 mM magnesium acetate, 60 mM potassium chloride, 10 mM 1,4-dithiothreitol, and the S-100 in 100 mM potassium phosphate buffer (pH 7.0) was incubated for 15 minutes at 37°C. The reaction mixture was chilled in an ice bath, and 200 μ l of the reaction mixture was passed through a column of Amberlite CG-50 (NH₄⁺ form, 0.5 ml), and the column was washed with 5 ml of water. The phosphorylated butirosin A on the column was eluted with 2 ml of 4 N ammonia into a scintillation vial and 8 ml of BRAY'S scintillator was added. The radioactivity (dpm) corresponding to the amount of the phosphorylated butirosin A was counted by a liquid scintillation system (Aloka LSC-653). The alkaline phosphatase activity was assayed by the method of GAREN *et al.*⁶⁾ One unit (U) was defined as the amount of the enzyme which hydrolysed 1 μ mole of *p*-nitrophenylphosphate per an hour. The activity of butirosins was determined by the cylinder plate method using *Escherichia coli* K12 ML1630 as the test organism and butirosin A as the standard.



carbonate, pH 7.4, at 28°C. Activities of intracellular phosphotransferase⁶⁾ and alkaline phosphatase⁷⁾, production of butirosins and pH of the cultured broth during fermentation are shown in Fig. 1. Starting on day 1 of the culture the phosphotransferase activity decreased gradually, while two maximum peaks of phosphatase activity were observed on days 2 and 5. The antibiotic activity began to appear in the cultured broth on day 6. Both the activity of the phosphotransferase and the alkaline phosphatase decreased during the production of butirosins.

To extract the phosphotransferase, cells of the 20-hour culture were harvested by centrifugation at $10,000 \times g$ for 10 minutes and washed with 20 mM potassium phosphate buffer (pH 7.2) containing 10 mM magnesium acetate, 60 mM potassium chloride and 10 mM 1,4-dithiothreitol. The washed cells were suspended in an equal volume of the buffer described above and disrupted by passage through a French pressure cell ($1,200 \text{ kg/cm}^2$). The homogenate was centrifuged at $100,000 \times g$ for 60 minutes, and the supernatant which contained the phosphotransferase was used as a crude enzyme solution (S-100).

The substrate specificity of this enzyme (S-100) was examined. As shown in Table 1, the enzyme (S-100) phosphorylated butirosin A, ribostamycin, kanamycins A and B, but not lividomycin A and this substrate requirement was the same as the APH (3')-II type in resistant bacteria. However, the *Bacillus circulans* enzyme was different from the latter in its behavior during affinity chromatography using kanamycin A-Sepharose 4B⁶⁾, since it did not bind to the affinity column. Moreover, the *Bacillus circulans* enzyme (S-100) did not produce the precipitin line against the rabbit antiserum of the APH(3')-II of *Escherichia coli* JR66/W677⁸⁾, and the enzymatic activity was not inhibited by the antiserum.

In order to determine the structure of the enzyme reaction product, the enzyme reaction was carried out in a mixture (150 ml) containing 75 mg of butirosin A, 750 mg of disodium adenosine triphosphate, 300 mg of sodium bicarbonate, 10 mM magnesium acetate, 60 mM potassium chloride, 10 mM 1,4-dithiothreitol and S-100 (200 mg protein) in 100 mM potassium phosphate buffer (pH 7.2). After 6-hour incubation at 37°C, the reaction mixture was diluted with 150ml of water and kept in a boiling water bath for 10 minutes. It was filtered and the filtrate was

Table 1. The substrate specificity of the phosphotransferase extracted from *Bacillus circulans*.

Antibiotic	Radioactivity (dpm)*
Butirosin A	6,178
Kanamycin A	961
Kanamycin B	3,217
Ribostamycin	6,150
Lividomycin A	80

The assay condition of the phosphotransferase is described in Fig. 1. The concentrations of the antibiotics added as the substrates were 0.2 mM.

* Radioactivity means dpm of the reaction products.

passed through a column of Amberlite CG-50 (NH_4^+ form, 15 ml). After washing with 150 ml of water, the phosphorylated butirosin A was eluted with 0.1 N ammonia. The eluate which showed positive ninhydrin and RYDON-SMITH reactions was concentrated to dryness yielding 58 mg of a yellowish powder. The powder was rechromatographed on a column of Amberlite CG-50 (NH_4^+ form, 10 ml), using 0.05 N ammonia for elution. The purified phosphorylated butirosin A was obtained as a white powder (40 mg). Reflux with 0.4 N hydrogen chloride in methanol for 2 hours gave ribose and a phosphate of 1-N-(4-amino-2-hydroxybutyryl)neamine. Hydrolysis of the phosphorylated butirosin A by alkaline phosphatase afforded butirosin A. Its identity with butirosin A 3'-phosphate²⁾ was confirmed by its PMR (D_2O , TMS as an external reference): the signal of the 3'-H at δ 4.1 in butirosin A shifted to δ 4.4 in the phosphorylated butirosin A.

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