INDIRECT INVOLVEMENT OF THE STRINGENT RESPONSE OF Bacillus Subtilis IN THE DEVELOPMENT OF SELF-RESISTANCE TO ITS OWN ANTIBIOTIC

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Bacillus subtilis Marburg has been found to produce an appreciable antibiotic activity in a synthetic medium, in parallel with its growth1). Using an isogenic relaxed mutant, which is not able to exert the stringent response (i.e. accumulation of intracellular ppGpp (guanosine 5'-diphosphate 3'-diphosphate) or pppGpp (guanosine 5'-triphosphate 3'-diphosphate)) due to a relA mutation, it has been reported that antibiotic production by B. subtilis is initiated by the stringent response1). In the present communication, we are reporting that the stringent response also initiates indirectly the resistance of B. subtilis to its own antibiotic (self-resistance).

The antibiotic produced in the medium (2 liters) was partially purified using a carbon column (200 ml): after washing with water (2 liters) the antibiotic was eluted with 60 % acetone. The fraction containing the antibiotic activity was concentrated by vacuum-evaporation to a volume of 20 ml. This preparation had an antibiotic activity of 12,000 units/ml, and was used for the following experiments. The molecular weight of this unidentified antibiotic was about 1,000 as estimated by ultrafiltration (Diafilter, Ulvac Co.).

Strain 61884 (aspB66 trpC2) was inoculated at low A600 (0.005) into flasks containing synthetic medium2), which contains excess glucose, ammonium sulfate, phosphate, and essential growth requirements (20 mM aspartate and 0.5 mM tryptophan). Cultures were grown using reciprocal shaking at 37°C. When the A600 reached 0.5 (middle exponential) various amounts of antibiotic were added, and the incubation was further continued for 4 hours. As Fig. 1 shows, the growth was markedly inhibited with the antibiotic at 150 units/ml or more. The decrease of A600 at 200 units/ml was due to the lysis of cells as was determined microscopically. The strain 61884 has an ability to produce the antibiotic activity of 25 units/ml in the synthetic medium under similar culture conditions2).

Next, we studied whether the stringent response initiates self-resistance or not. The strain 61884, grown in synthetic medium with excess aspartate (20 mM) to A600=0.5, was harvested on a Millipore filter, washed and immediately transferred to a similar medium containing a limiting amount (2 mM) of aspartate (Fig. 2A). Cells grew slowly with the insufficient (but continuous) supply of aspartate (due to the uptake competition with glutamate in the medium2)). Such conditions caused a partial stringent response: intracellular ppGpp transiently increased from 4 pmol/AM500 to 90 pmol/AM500 10 minutes after cell transfer, then gradually decreased to a level still higher than the initial one2). Two hours after this imposition of aspartate limitation growth was no longer

Fig. 1. Effect of antibiotic on B. subtilis strain 61884 (aspB66 trpC2) growing with excess requirements.

Experimental procedures were described in the text or in previous report1). The numbers on the curves indicate the concentration of antibiotic (units/ml).
affected by the antibiotic (1,000 units/ml) (Fig. 2A). [Strain 61884 is able to produce the antibiotic activity of 90 to 120 units/ml 5 hours after cell transfer to aspartate-limiting medium1]. If the antibiotic was added immediately after cell transfer growth was still markedly inhibited by 150 units/ml or more of antibiotic (data not shown). These results suggest that the cells need a finite time period to make them resistant to the antibiotic.

It is believed that ppGpp (or pppGpp) is responsible for the stringent response which affects many widespread cellular reactions. To determine whether or not an accumulation of ppGpp or pppGpp per se was responsible for the initiation of self-resistance, we used the fact that 1 mg/ml of chloramphenicol interfered with the bacterial synthesis of both ppGpp and pppGpp without appreciably affecting the growth rate3). Under these conditions antibiotic production was greatly reduced1). Also, as shown by Fig. 2B, addition of chloramphenicol (1 mg/ml) into the transfer medium drastically reduced the self-resistance of the B. subtilis. However, addition of small amounts (100 units/ml) of antibiotic into the transfer medium did almost completely restore the self-resistance (Fig. 2C). Moreover, a relaxed mutant strain 61883 (aspB66 trpC2 relA1), unable to produce both ppGpp and pppGpp due to relA mutation3), could develop self-resistance comparable to that of strain 61884 if small amount (100 units/ml) of antibiotic was added into the transfer medium (not shown). Thus, prior synthesis of antibiotic (induced by the stringent response) seems responsible for the initiation of self-resistance, but not by a direct involvement of the stringent response. In contrast, the stringent response was indispensable for the initiation of antibiotic production of B. subtilis: relaxed mutants (relA and relC) were no longer able to produce antibiotic in any nutrient media (checked with ten kind of media with varying carbon or nitrogen sources).

In actinomycetes, the self-resistance mechanisms have been studied with respect to membrane permeability, enzymatic inactivation or ribosome sensitivity4~9). A-Factor, a metabolite of certain actinomycetes, has been shown to control the self-resistance10), in addition to the initiation of antibiotic production. The mechanism of development of the self-resistance by the producing antibiotic is now under investigation.

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References


7) SKINNER, R. H. & E. CUNDLIFE: Resistance to the antibiotics viomycin and capreomycin in the Streptomyces species which produce them. J. Gen. Microbiol. 120: 95~104, 1980

