INDIRECT INVOLVEMENT OF THE STRINGENT RESPONSE OF *BACILLUS SUBTILIS* IN THE DEVELOPMENT OF SELF-RESISTANCE TO ITS OWN ANTIBIOTIC

KOZO OCHI and MICHIO YAMASHITA

Exploratory Research Laboratories
Fujisawa Pharmaceutical Co., Ltd.
2-1-6 Kashima, Yodogawa-ku, Osaka 532, Japan

(Received for publication May 30, 1984)

Bacillus subtilis Marburg has been found to produce an appreciable antibiotic activity in a synthetic medium, in parallel with its growth[1]. 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 response[1]. 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 A_{600} (0.005) into flasks containing synthetic medium[2], 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 A_{600} 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 A_{600} 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 conditions[1].

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 A_{600}=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 medium[2]). Such conditions caused a partial stringent response: intracellular ppGpp transiently increased from 4 pmol/AM_{600} (absorbancy mass) to 90 pmol/AM_{600} 10 minutes after cell transfer, then gradually decreased to a level still higher than the initial one[1]. 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 report[1]. The numbers on the curves indicate the concentration of antibiotic (units/ml).

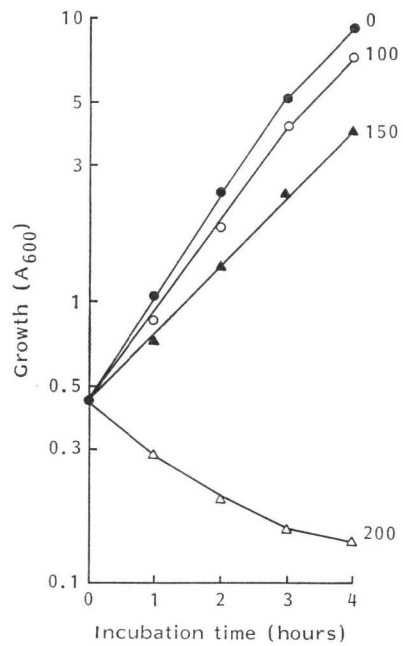
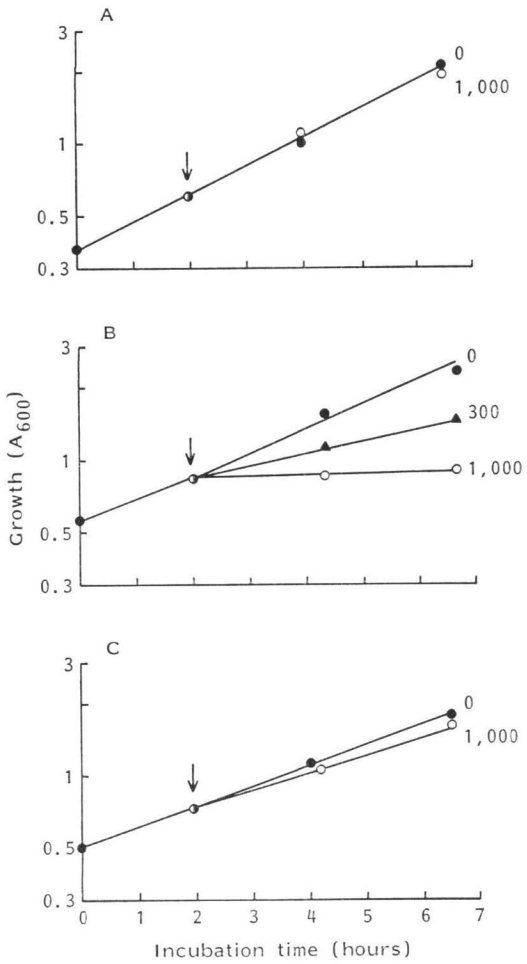


Fig. 2. Effect of antibiotic on *B. subtilis* strain 61884 growing with limited supply of aspartate.

Experimental procedures were same as Fig. 1 except that cells were transferred from the medium containing excess (20 mM) aspartate to limiting aspartate (2 mM) (see text).

Arrows and numbers on the curves indicate the addition of antibiotic and its concentration (●, 0 unit/ml: ▲, 300: ○, 1,000), respectively.

A: Growth after cell transfer to aspartate-limiting medium. B: Chloramphenicol (1 μg/ml) was added to transfer medium at 0 hour. C: Chloramphenicol (1 μg/ml) and antibiotic (100 units/ml) were added to transfer medium at 0 hour.



affected by the antibiotic (1,000 units/ml) (Fig. 2A). [Strain 61884 is able to produce the antibiotic activity of 90~120 units/ml 5 hours after cell transfer to aspartate-limiting medium[1]]. 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 μg/ml of chloramphenicol interferred with the bacterial synthesis of both ppGpp and pppGpp without appreciably affecting the growth rate[3]. Under these conditions antibiotic production was greatly reduced[1]. Also, as shown by Fig. 2B, addition of chloramphenicol (1 μg/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* mutation[2], 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 sensitivity[4~9]. A-Factor, a metabolite of certain actinomycetes, has been shown to control the self-resistance[10], in addition to the initiation of antibiotic production. The mechanism of development of the self-resistance by the producing antibiotic is now under investigation.

Acknowledgment

We express our thanks to Drs. H. AOKI and M. KOHSAKA for their permission to submit the manuscript.

References

1) OCHI, K. & S. OHSAWA: Initiation of antibiotic production by the stringent response of *Bacillus subtilis* Marburg. J. Gen. Microbiol., in press
2) OCHI, K.; J. C. KANDALA & E. FREESE: Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. J. Biol. Chem. 256: 6866~6875, 1981
3) OCHI, K. & E. FREESE: Effect of antibiotics on sporulation caused by the stringent response in *Bacillus subtilis*. J. Gen. Microbiol. 129: 3709~3720, 1983
4) CELLA, R. & L. C. VINING: Resistance to streptomycin in a producing of *Streptomyces griseus*. Can. J. Microbiol. 21: 463~472, 1975
5) GRAHAM, M.Y. & B. WEISBLUM: 23S ribosomal ribonucleic acid of macrolide-producing streptomycetes contains methylated adenine. J. Bacteriol. 137: 1464~1467, 1979
6) PIWOWARSKI, J. M. & P. D. SHAW: Streptomycin resistance in a streptomycin-producing microorganisms. Antimicrob. Agents Chemother. 16: 176~182, 1979
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
8) SUGIYAMA, M.; H. KOBAYASHI, O. NIMI & R. NOMI: Susceptibility of protein synthesis to streptomycin in streptomycin-producing *Streptomyces griseus*. FEBS Lett. 110: 250~252, 1980
9) HOTTA, K.; H. YAMAMOTO, Y. OKAMI & H. UMEZAWA: Resistance mechanisms of kanamycin-, neomycin-, and streptomycin-producing streptomycetes to aminoglycoside antibiotics. J. Antibiotics 34: 1175~1182, 1981
10) HARA, O. & T. BEPPU: Induction of streptomycin-inactivating enzyme by A-factor in *Streptomyces griseus*. J. Antibiotics 35: 1208~1215, 1982