STUDIES ON BACTERIAL CELL WALL INHIBITORS

VIII. MODE OF ACTION OF A NEW ANTIBIOTIC, AZUREOMYCIN B, IN *BACILLUS CEREUS* T*

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(Received for publication July 11, 1979)

Azureomycin B, a new antibiotic which contains sugar, amino acid and phenol moieties and inhibits Gram-positive bacteria, was found to be a specific inhibitor of peptidoglycan synthesis in bacteria. The antibiotic lysed growing cells of *Bacillus cereus* T at a concentration of 10 μg/ml but did not affect resting cells. Microscopical observation revealed swelling and lysis of the bacterial rods when treated with azureomycin B. The incorporation of [^{3}H]diaminopimelic acid or [^{14}C]glucosamine into acid-insoluble fraction of growing cells of *Bacillus cereus* T was strongly inhibited in the presence of azureomycin B, but that of [^{14}C]leucine, [^{3}H]thymidine or [^{3}H]uridine were not, at least until 5 minutes after the beginning of the incubation. The antibiotic caused accumulation of a nucleotide precursor in the cells which was identified as UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala. Thus the site of action was suggested to lie between this nucleotide and peptidoglycan in the pathway of peptidoglycan synthesis.

In the course of our screening research for new antibiotics inhibiting bacterial cell wall synthesis[1], *Pseudonocardia azurea* nov. sp., a soil isolate secreting a characteristic blue pigment when grown on a glucose-nitrate agar, was found to produce new antibiotics, azureomycins A and B. Characteristics of the producing organism, the isolation of azureomycins A and B, as well as their biological and physico-chemical properties have been reported in a preceding paper[2]. The antibiotics contain sugar, amino acid and phenol moieties and inhibit Gram-positive bacteria including *Mycobacterium* and *Clostridium*.

In the present paper more detailed data on its mode of action are reported, which confirm the inhibitory effect of azureomycin B on cell wall peptidoglycan synthesis and indicate that the site of action lies after UDP-MurNAc-pentapeptide in the biosynthetic pathway of peptidoglycan.

Materials and Methods

Bacterial strain

Bacillus cereus T which was obtained from Dr. K. IZAKI, School of Agriculture, Tohoku Univer-

* Part VII appears in Ref. 2. This work was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan.

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Abbreviations: UDP-MurNAc-pentapeptide, uridine-diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine; UDP-MurNAc, uridine-diphosphate-N-acetylmuramic acid; Dpm, diaminopimelic acid; GlcNAc, N-acetylglucosamine.

sity, Sendai, Japan, and kept on heart infusion agar, was used.

Growth of bacterial strain

B. cereus T was grown in nutrient broth (1% peptone, 1% meat extract, 0.5% NaCl, pH 7.2) at 37°C. In small scale experiments (up to 10 ml culture volume) a Jasco Bio-Log II photometer and a Monod Shaker were used for the incubation. Large-scale growth was done with a reciprocal shaker using SAKAGUCHI's flasks. For viable count, an aliquot of the growing culture in biophotometer cells was diluted with water, spread on a nutrient agar plate and incubated at 37°C overnight.

Microscopical investigation

B. cereus T was grown in nutrient broth at 37°C (5 ml) in a Monod tube until the absorbance at 660 nm reached 0.4 (the absorbance was measured with a Coleman Junior II Spectrophotometer). Then azureomycin B was added to the culture to give a final concentration of 20 μg/ml and the culture was incubated for additional 30 minutes. A culture without antibiotic served as control. Then a loopful of the cultures (control and antibiotic-treated one) was transferred to microscope slides, stained with PFEIFFER's solution and observed with an Olympus Vanox photomicroscope.

Incorporation of [^{3}H]Dpm, [^{14}C]glucosamine, [^{3}H]thymidine, [^{3}H]uridine and [^{14}C]leucine into acid-insoluble macromolecular fraction of growing cells of *B. cereus* T

B. cereus T was incubated in nutrient broth until the absorbance at 660 nm reached 0.2. Azureomycin B and [^{3}H]thymidine (2 μCi), [^{3}H]uridine (0.5 μCi), [^{14}C]leucine (2 μCi), 2,6-[^{3}H]Dpm (0.2 μCi) or [^{14}C]glucosamine (2 μCi) was added to 1.4 ml of the culture in the case of [^{3}H]thymidine incorporation and to 1.6 ml in the latter ones. Cold thymidine solution (0.2 ml of a 1 μg/ml solution) was then added to the reaction mixture containing [^{3}H]thymidine. The reaction mixtures, total volume 2 ml each, were incubated in Monod tubes. After 5, 10, 15 and 20 minutes, 0.4 ml aliquots of the cultures were put into 10 ml of ice-cold 5% trichloroacetic acid, the resulting acid-insoluble precipitates were collected on a Millipore filter (pore size 0.22 μ) and washed twice with 10 ml of cold 5% trichloroacetic acid. The radioactivities of the acid-insoluble fractions on the filters were counted with 10 ml of a liquid scintillation fluid (4 g 2,6-diphenyloxazole, 100 mg 1,4-bis-(2-(5-phenyloxazolyl))benzene in 1 liter toluene) using an Aloka LSC-651 scintillation counter.

Peptidoglycan synthesis and nucleotide accumulation in a growing culture

For the time-course experiment of peptidoglycan synthesis and nucleotide accumulation, *B. cereus* T was incubated in 4.5 ml nutrient broth in a Monod tube until the absorbance at 660 nm reached 0.6 (half maximal growth). Then 2,6-[^{3}H]Dpm (5 μCi) and azureomycin B (50 μg/ml) were added to give a total volume of 5 ml. After 10, 30 and 60 minutes, 0.2-ml aliquots of the culture were put into 10 ml of ice-cold 5% trichloroacetic acid, the acid-insoluble precipitates were collected on a Millipore filter and the radioactivity was counted (corresponding to peptidoglycan synthesis). At the same time, 1-ml aliquots of the culture were centrifuged for 10 minutes at $1,500 \times g$ at 4°C. The pellets were washed twice with water, suspended in 1 ml water, and heated for 2 minutes in a boiling water bath. The heated suspensions were centrifuged for 10 minutes at $1,500 \times g$, and 0.2 ml aliquots of the supernatants were chromatographed on Toyo Roshi No. 50 paper (1×40 cm) with isobutyric acid - 1 N ammonia (5: 3, v/v) for 16 hours. The paper strips were cut into 1×2 cm pieces and the radioactivities were counted in the toluene scintillation system described above. In the above solvent system the Rf-value of UDP-MurNAc-peptides is about 0.15.

Identification of the nucleotides accumulated in the cells

B. cereus T was incubated in 1 liter nutrient broth and distributed to 100 ml in SAKAGUCHI's flasks. At half maximal growth of the culture, (absorbance at 660 nm was 0.6) 500 ml of it was treated with azureomycin B (20 μg/ml) for 30 minutes. The nontreated half served as control. Extraction of the nucleotide precursor, anion-exchange chromatography and determination of GlcNAc as N-acetylhexosamine were carried out according to STROMINGER[3] and REISSIG *et al.*[4] Amino acid analysis was done using a Jeol JL C-6 AH amino acid analyzer.

Chemicals

Azureomycin B was isolated as described by S. ŌMURA *et al.*[2] Chloramphenicol was obtained

from Sankyo Co., Ltd. (Japan), rifampicin and actinomycin D were received from Daiichi Seiyaku Co., Ltd. (Japan). Other chemicals used were reagent grade.

Radiochemicals

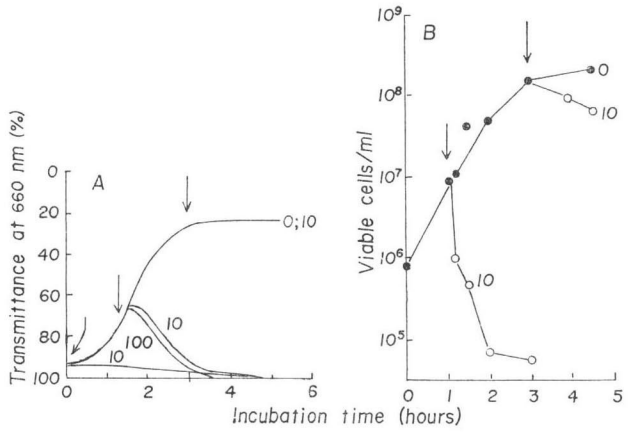
(DL+*meso*)-2,6-Diamino[6-^{3}H]pimelic acid dihydrochloride (1.5 Ci/mmole), L-[U-^{14}C]leucine (345 mCi/mmole), [6-^{3}H]thymidine (21.5 Ci/mmole), [^{3}H]uridine (21.5 Ci/mmole) and [^{14}C]glucosamine (56.6 mCi/mmole) were obtained from the Radiochemical Centre Amersham, U. K.

Results

Effect of Azureomycin B on the Growth of *B. cereus* T

When azureomycin B was added to the culture medium before incubation, it inhibited the growth of *B. cereus* T at a concentration of 10 μg/ml (Fig. 1A). Upon its addition (10 μg/ml) during the log phase of the culture, the growth observed as a function of decreasing light transmission seemed to continue for about 15 minutes, and then lysis occurred. The apparent continuation of growth may be due to swelling of the cells before lysis, resulting in a decrease of light transmission. When the viable cells in the same culture were counted, a considerable decrease was already observed after 10 minutes of incubation with azureomycin B (10 μg/ml) (Fig. 1B). Upon addition of azureomycin B at the beginning of the stationary growth phase, no or only a weak effect on growth (Figs. 1A and 1B) was seen. This observation is common to cell wall synthesis inhibitors which act only on growing but not on resting cells.

Fig. 1. Effect of azureomycin B on (A) growing culture and on (B) the viability of cells of *B. cereus* T in nutrient broth at 37°C.
The arrows indicate the addition periods of azureomycin B. The numbers show the concentrations (μg/ml).



Morphological Change of Cells Treated by Azureomycin B

Plate 1 shows normal cells of middle log phase of *B. cereus* T as revealed by photomicroscopy. Plate 2 shows the cells under the influence of treatment with azureomycin B (20 μg/ml) for 30 minutes. Compared to the control, the azureomycin B-treated cells appear swollen. The swollen cells then lysed (Plate 2, indicated with arrows).

Effect of Azureomycin B on the Biosynthesis of Protein, Nucleic Acids and Cell Wall Peptidoglycan in *B. cereus* T

The incorporation of labeled precursors of protein, DNA, RNA and peptidoglycan (L-[^{14}C]leucine, [^{3}H]thymidine, [^{3}H]uridine, [^{3}H]Dpm and [^{14}C]glucosamine, respectively) into acid-insoluble macromolecular material, was studied in the presence of azureomycin B. Fig. 2 shows that the incorporation of [^{14}C]leucine, [^{3}H]thymidine and [^{3}H]uridine was not inhibited in the presence of 10 μg/ml of azureomycin B for at least 5 minutes, whereas typical inhibitors of protein, DNA and RNA syntheses like

Plate 1. Photomicrograph of middle log phase cells of *B. cereus* T (control).

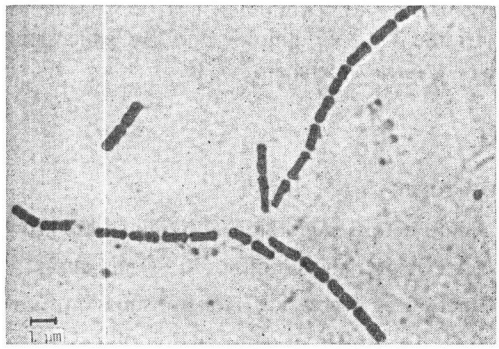


Plate 2. Photomicrograph of middle log phase cells of *B. cereus* T treated by azureomycin B (20 μg/ml) for 30 minutes.
The arrows indicate lysed cells.

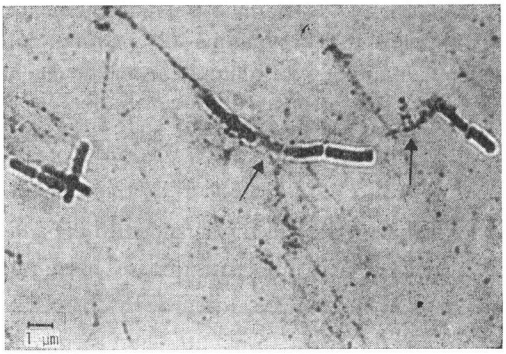
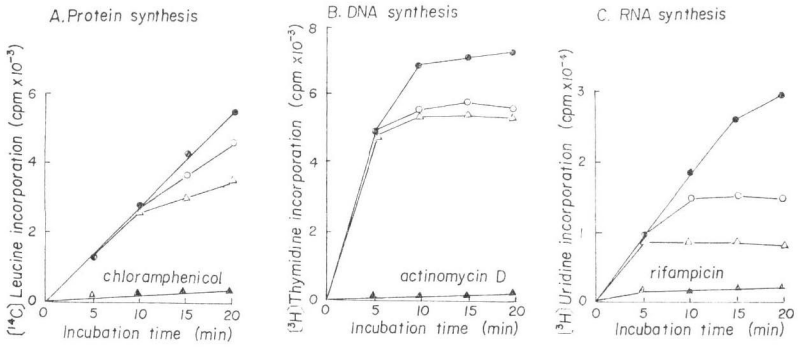
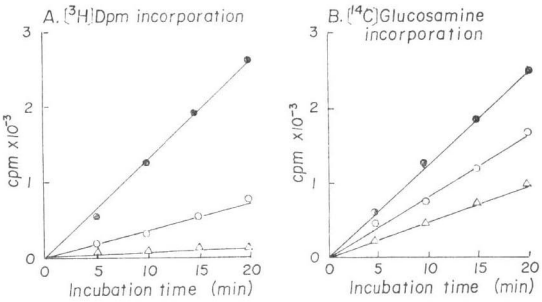


Fig. 2. Effect of azureomycin B on the incorporation of [^{14}C]leucine (A), [^{3}H]thymidine (B) and [^{3}H]uridine (C) into acid-insoluble fraction in *B. cereus* T in comparison with chloramphenicol (100 μg/ml), actinomycin D (10 μg/ml) and rifampicin (10 μg/ml), respectively. ●, control; ○, 10 μg/ml azureomycin B; △, 50 μg/ml azureomycin B.



chloramphenicol, actinomycin D and rifampicin, respectively, inhibited the incorporation of precursors from the beginning of the incubation. The inhibition observed after 10~20-minute incubation is to be regarded as secondary effect derived from cease of growth and lysis of the cells which was caused by the interference of azureomycin B with peptidoglycan synthesis as shown in Fig. 3. This figure shows that the incorporation of [^{3}H]Dpm and [^{14}C]glucosamine into acid-insoluble macromolecular fraction of *B. cereus* T was inhibited from the beginning in the presence of 10 μg/ml of azureomycin B, the concentration which inhibited the growing culture of the organism (see Fig. 1). For 10 μg/ml azureomycin B, the extent of inhibition of [^{3}H]Dpm incorporation was 63%, and 35% in the case

Fig. 3. Effect of azureomycin B on the incorporation of [^{3}H]Dpm (A) and [^{14}C]glucosamine (B) into acid-insoluble macromolecular fraction in *B. cereus* T. ●, control; ○, 10 μg/ml azureomycin B; △, 50 μg/ml azureomycin B.

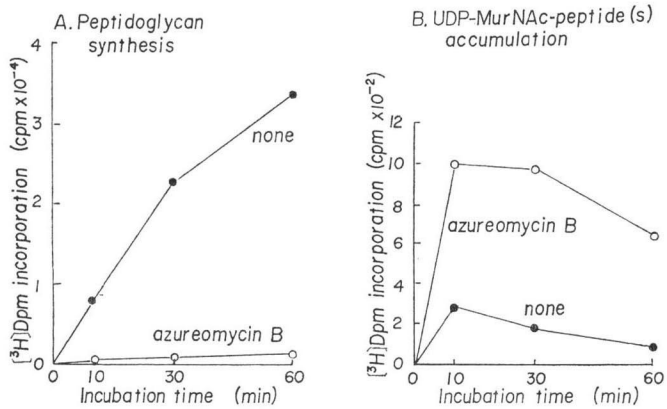


of [^{14}C]glucosamine incorporation. The weaker inhibition of [^{14}C]glucosamine incorporation can be interpreted as indicating that azureomycin B affects only peptidoglycan synthesis because Dpm is a more specific precursor for it, whereas glucosamine can be easily metabolized and incorporated into other macromolecules.

Time Course of UDP-MurNAc-peptide(s) Accumulation and Peptidoglycan Synthesis in the Absence and Presence of Azureomycin B in a Growing Culture of *B. cereus* T

Fig. 4A shows peptidoglycan synthesis in the absence and presence of azureomycin B (50 μg/ml) determined as the incorporation of [^{3}H]Dpm into acid-insoluble fraction of *B. cereus* T collected on a Millipore filter. In Fig. 4B UDP-MurNAc-peptide accumulation both with and without azureomycin B (50 μg/ml) is seen which was determined after paper chromatographic separation of the cell extract from an aliquot of the same culture as that used in Fig. 4A. When the organism was incubated in the presence of azureomycin B, peptidoglycan synthesis from [^{3}H]Dpm was strongly inhibited (Fig. 4A) and UDP-MurNAc-peptide(s), precursor(s) of peptidoglycan, accumulated in the cells (Fig. 4B).

Fig. 4. Time course of peptidoglycan synthesis (A) and UDP-MurNAc-peptide(s) accumulation (B) by *B. cereus* T in the absence and presence of azureomycin B (50 μg/ml).



Identification of the Nucleotide Precursor(s) Accumulated in the Cells

Fig. 5 shows the column chromatograms of cold trichloroacetic acid extracts from nontreated cells (I) and from cells incubated with azureomycin B (II). A large peak (fraction numbers 95 and 96) eluted with 0.1 M NaCl in 0.01 N HCl was obtained only in the antibiotic-treated culture. Analysis of the UV-absorbing substance in this peak indicates that it contains uridine, N-acetylhexosamine, glutamic acid, diaminopimelic acid and alanine in a molar ratio of about 1 : 1 : 1 : 1 : 3 (Table 1). This suggests that the accumulated substance is UDP-MurNAc-L-Ala-

Table 1. Analysis of the accumulated nucleotide (fractions 95 and 96).

The fractions were combined and further purified by charcoal adsorption and elution and by paper chromatography, and then analyzed. The analytical methods were: uridine, spectrophotometrically; GlcNAc, as N-acetylhexosamine described by REISSIG *et al.*[4] and STROMINGER[3]; amino acids, by amino acid analyzer after acid hydrolysis.

Component	μmoles/ml (molar ratio)
Uridine	0.042 (1.00)
N-Acetylhexosamine	0.047 (1.12)
Glutamic acid	0.038 (0.91)
Diaminopimelic acid	0.032 (0.76)
Alanine	0.114 (2.71)

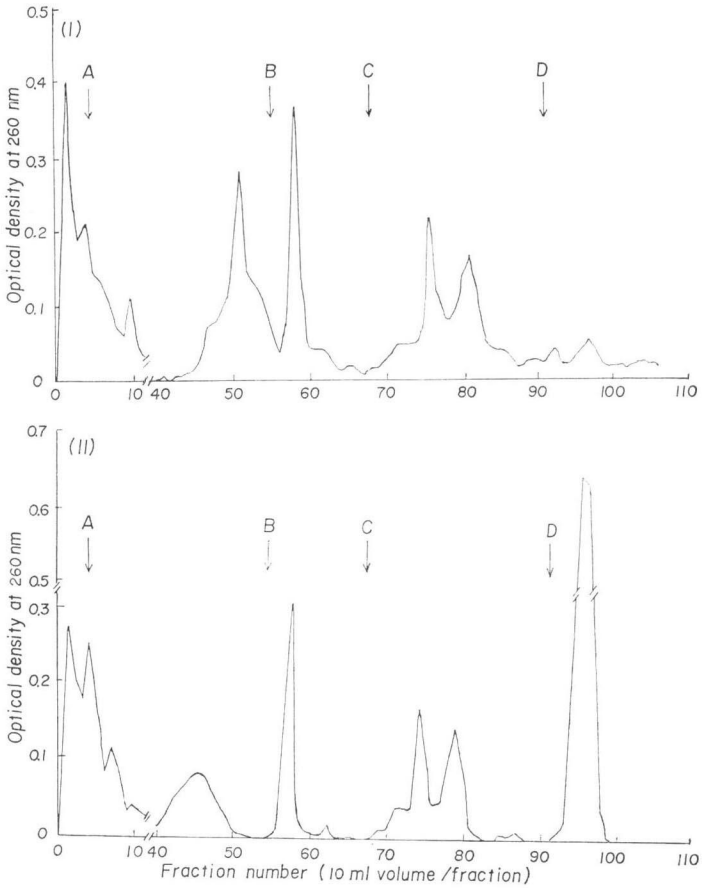
D-Glu-*meso*-Dpm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide).

Discussion

The swelling and bactericidal effects of azureomycin B on *B. cereus* T, the selective inhibition of incorporation of peptidoglycan precursors in the presence of the antibiotic and the accumulation of UDP-MurNAc-pentapeptide in azureomycin B-treated cells provide evidence for peptidoglycan synthesis as the target of this new antibiotic. Accumulation of uridine nucleotide precursors in the cells is known for many other cell wall synthesis inhibitors like penicillin[3], vancomycin[5,6], amphomycin[7], enduracidin[8], gardimycin[9] and moenomycin-group antibiotics[10,11]. Because UDP-MurNAc-pentapeptide was accumulated in the presence of azureomycin B, a biosynthetic step after the formation of this precursor must be inhibited by the antibiotic. To clarify the site of action of azureomycin B, further *in vitro* studies are necessary which will show whether the antibiotic interfers with the formation of lipid intermediates (the membrane-bound precursors of peptidoglycan), with the polymerization of these precursors to linear glycan strands or with the cross-linking reaction of glycopeptide chains.

Fig. 5. Anion-exchange chromatograms of cold trichloroacetic acid extracts from cells of *B. cereus* T. The extract obtained from 500 ml each of a control culture (I) and an azureomycin B-treated (20 μg/ml) culture (II) were applied to a Dowex-1 × 2 (Cl^-) column (15 cm[3]).

Elution was carried out as described by STROMINGER[3]. Eluant: A, 0.002 N HCl; B, 0.01 N HCl; C, 0.05 M NaCl in 0.01 N HCl; D, 0.1 M NaCl in 0.01 N HCl.



Acknowledgement

The authors wish to thank Mr. H. HIGUCHI of Tokyo Research Laboratories, Kyowa Hakko Kogyo, Co., Ltd., for the amino acid analysis.

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