BACTERIAL LYSIS OF *CLOSTRIDIUM* SPECIES
III. PROPERTIES OF PREMATURE LYSIS PROVOKED
BY ANTIBIOTICS IN MITOMYCIN C-INDUCED LYSIS
OF *CLOSTRIDIUM SACCHAROPERBUTYLACETONICUM*¹

SEIYA OGATA, KYOUNG HO CHOI, YOSHIFUMI IKEDA,²
AND MOTOYOSHI HONGO

*Laboratory of Applied Microbiology, Department of Agricultural Chemistry,
Kyushu University, Fukuoka 812*

(Received March 25, 1974)

Clostridium saccharoperbutylacetonicum was induced to lyse by exposure to mitomycin C, with a concomitant production of bacteriocin (clostocin O). The mature lysis began about 180 min after mitomycin C treatment and completed after 5 hr or more. By the treatment with various antibiotics, premature lysis was provoked before 180 min, as follows: 1) Chloramphenicol, oxytetracycline and many other antibiotics inhibited the induction of lysis, when added during the first 90 min, by preventing the synthesis of clostocin O-endolysin. However, when they were added at a later period, at which the active endolysin was apparently synthesized in the organisms, they provoked premature lysis of the organisms. The premature lysis began 5 to 30 min after the addition of antibiotics, and finished after about 60 min, when the correct concentration of antibiotics was used. The lag period after addition of antibiotics before lysis began differed from one antibiotic to another. The rate of lysis was accelerated according to the increased content of endolysin and to the increased concentration of antibiotics. Among the many antibiotics used, protein synthesis inhibitors were the most effective in inducing premature lysis. In the case of oxytetracycline-resistant mutant, the premature lysis was not provoked by oxytetracycline, but was by chloramphenicol, *etc.* From these results it was considered that premature lysis of mitomycin C-treated organisms was due both to the action of clostocin O-endolysin and to some metabolic perturbation of protein synthesis resulting from antibiotic treatment.

2) Penicillin, cephaloridine, and cephalosporin C did not inhibit

¹ A part of this work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, held at Tokyo, April 1, 1971.

² Present address: Yoshitomi Laboratory, Yoshitomi Pharm. Ind. Co., Ltd. Yoshitomi-cho, Fukuoka 871.

the synthesis of endolysin. Their action on the premature lysis was very different from those of many other antibiotics described above. Mitomycin C-treated organisms were provoked by them to lyse prematurely even when added early in the induction process. However, the actual lysis did not begin until the active endolysin appeared in the organisms, because the content of active endolysin originating from the normal organisms became very small after mitomycin C treatment.

It was concluded that the inhibition of cell wall synthesis or its related protein synthesis resulted in premature lysis in the presence of clostocin O-endolysin. It was further discussed that the mature lysis might be determined by the decline in wall synthesis in addition to the action of endolysin.

It is known that the production of phage or inducible bacteriocin and the release of these particles are accompanied by the lysis of producer organisms (1-7). This lysis is produced by the breakdown of cell wall by endolysin (endolytic enzyme) synthesized after phage infection or bacteriocin induction. The relation of endolysin synthesis to cellular lysis was reported in some organisms infected by phage such as *E. coli* phage λ (8-10) and *Salmonella* phage P 22 (11, 12). These observations support the hypothesis that the timing and rate of endolysin synthesis determine the kinetics of the lytic process. These workers also show that some factors besides endolysin have an effect on the lytic process but, until now, the lytic process has been obscure in detail. Moreover, little work has been done to elucidate the lytic process for bacteriocin production.

In the previous paper, we reported the mitomycin C-induced lysis of *Clostridium saccharoperbutylacetonicum*, with a concomitant production of a bacteriocin (clostocin O). The mature lysis began about 180 min after mitomycin C treatment, and completed 5 hr or more later. Active clostocin O and its endolysin appeared in the organisms about half-way of the latent period of induction process, and their synthesis terminated about 30 min before the mature lysis (13). The much longer latent period may be a specific difference discerned between our organisms and many other organisms reported. This specificity may present some clues for the analysis of the mechanisms of cellular lysis caused by phage infection or bacteriocin induction, because each step of lytic process of our organisms is slow.

This paper describes the properties of premature lysis provoked by antibiotic treatment in the mitomycin C-induced lysis. Several hypotheses for the feature of mitomycin C-induced lysis are also presented from the results obtained.

MATERIALS AND METHODS

Organisms. The strain used was NI-4 (ATCC 13564) of *Clostridium sac-*

charoperbutylacetonicum previously found to produce an inducible phage tail-like bacteriocin, clostocin O (13). An oxytetracycline-resistant mutant was obtained from the sensitive parent strain NI-4 by one-step isolation method in the presence of 30 $\mu\text{g/ml}$ of oxytetracycline (14).

Medium and cultural conditions. Growth was at 30° under reduced atmospheric pressure (5–10 mmHg) in TYA medium (15). To obtain a young exponentially growing culture, fresh medium was inoculated with sufficient organisms to produce an initial optical density (OD) of 0.1 at 660 nm and, unless otherwise mentioned, the culture was incubated until its OD₆₆₀ became 0.25 (ca. 8 to 10 $\times 10^7$ cells/ml).

Turbidity measurements. Optical density (OD) of the culture, cell and cell wall suspension, was measured at 660 nm with a photoelectric colorimeter (Model 7A, Tokyo Kodens Co., Ltd. or Type-101, Hitachi Co., Ltd.).

Lysis induction by mitomycin C treatment. The culture grown anaerobically at 30° to OD₆₆₀ of 0.25 was treated for 15 min at 30° with 4 $\mu\text{g/ml}$ mitomycin C (13). Excess mitomycin C was removed by centrifugation for 5 min at 9,000 $\times g$. The harvested organisms were suspended in prewarmed TYA broth to an OD₆₆₀ of 0.25, and the culture was incubated in a usual manner with reading of OD₆₆₀ made at 30-min intervals.

Antibiotic treatment. The known 36 antibiotics listed in Table 1 were used. Antibiotic treatment was performed on the normal growing and mitomycin C-treated cultures at a suitable period, and changes in the OD₆₆₀ of these cultures were monitored for 60 min or more.

Preparation of cell wall and Formalin-treated organisms. The cell wall and Formalin-treated organisms of strain NI-4 were prepared as described in previous papers (16, 17). The isolated cell wall was freeze-dried. Formalin-treated organisms were suspended in 0.067 M phosphate buffer (pH 5.5).

Assay of clostocin O-endolysin. Samples were withdrawn at 30-min intervals from a mitomycin C-treated culture. A portion of each sample was immediately centrifuged at 9,000 $\times g$ for 10 min, and the supernatant was assayed for extracellular activity of the endolysin. Another portion was exposed to 0.3 M NaCl at 30° until lysis was completed, when the lysate was centrifuged at 60,000 $\times g$ for 60 min to remove clostocin O, and the supernatant was assayed for total (intracellular and extracellular) endolysin activity; the lysis of strain NI-4 by NaCl treatment was described in the previous papers (15, 18).

Lytic activity was assayed by the decrease in turbidity of suspension of Formalin-treated organisms or isolated cell wall. The sample (1 ml) and 4 ml of substrate were suspended in 0.067 M phosphate buffer (pH 5.5) to an initial OD₆₆₀ of 0.50; phosphate buffer of pH 5.5 was used, since the pH of the lysate was about 5.5. A control tube contained a sample boiled for 5 min. Units were calculated from the following equation: units = $1,000 \times \Delta\text{OD}/\text{min}$. One unit for the digestion of cell wall was equivalent to 1.2 to 1.5 units for cellular lysis of Formalin-treated organisms.

Table 1. List of antibiotics used.

Name of antibiotic	Manufacturer or Dealer
Actinomycin D	Sigma Chem. Co.
Actinomycin S ^a	Takeda Chem. Ind. Co., Ltd.
Antimycin A	Seikagaku Kogyo Co., Ltd.
Bacitracin	Sigma Chem.
Bleomycin	Nippon Kayaku Co., Ltd.
Carzinophilin A	Kyowa Hakko Kogyo Co., Ltd.
Cephaloridine	Torii Pharm. Co., Ltd. (Glaxo Co.)
Cephalosporin C ^a	Takeda Chem. Ind. Co., Ltd.
Chloramphenicol	Sankyo Co., Ltd.
Chlortetracycline ^a	Takeda Chem. Ind. Co., Ltd. (Lederle Japan Co.)
Chromomycin A ₃ ^a	Takeda Chem. Ind. Co., Ltd.
Colistin	Banyu Pharm. Co., Ltd.
Cycloheximide ^a	Takeda Chem. Ind. Co., Ltd.
Cycloserine ^b	Kyowa Hakko Kogyo Co., Ltd.
Dihydrostreptomycin	Takeda Chem. Ind. Co., Ltd.
Erythromycin	Sankyo Co., Ltd. (Eli Lilly Co.)
Fradiomycin (Neomycin)	Takeda Chem. Ind. Co., Ltd.
Kanamycin	Takeda Chem. Ind. Co., Ltd.
Lateriomycin A ^a (Daunomycin-like)	Takeda Chem. Ind. Co., Ltd.
Leucomycin	Toyo Jozo Co., Ltd.
Mikamycin A ^c	Kanegafuchi Chem. Ind. Co., Ltd.
Mikamycin B ^c	Kanegafuchi Chem. Ind. Co., Ltd.
Mitomycin C ^b	Kyowa Hakko Kogyo Co., Ltd.
Novobiocin ^a	Takeda Chem. Ind. Co., Ltd.
Oleandomycin ^a	Takeda Chem. Ind. Co., Ltd.
Oxytetracycline	Pfizer Taito Co., Ltd.
Penicillin G	Meiji Seika Co., Ltd.
Polymyxin B	Pfizer Taito Co., Ltd.
Rifampicin	Pfizer Taito Co., Ltd.
Sarcomycin ^a	Banyu Pharm. Co., Ltd.
Spiramycin ^b	Kyowa Hakko Kogyo Co., Ltd.
Streptomycin	Toyo Jozo Co., Ltd.
Thiophenicol ^d	Eizai Co., Ltd.
Tolypomycin Y ^a (Rifampicin-like)	Takeda Chem. Ind. Co., Ltd.
Viomycin ^a	Takeda Chem. Ind. Co., Ltd.

^a These antibiotics were a gift from the Takeda Chemical Ind. Co., Ltd.

^b Kyowa Hakko Kogyo Co., Ltd.

^c Kanegafuchi Chemical Ind. Co., Ltd.

^d Eizai Co., Ltd.

Assay of autolysin. The lysis of mitomycin C-treated organisms was provoked by NaCl, as described above. The lysate was centrifuged at $9,000 \times g$ for 10 min to remove cell debris, and the supernatant was assayed for autolysin as described above. This experiment was performed in order to measure the autolysin activity remaining after mitomycin C treatment. The isolated cell wall suspended in

phosphate buffer of pH 5.5 was only used as the substrate in this experiment. Clostocin O-endolysin was clearly different from autolysin in its substrate specificity. The former easily digested Formalin-treated organisms as well as isolated cell wall, while the latter was hard to digest Formalin-treated organisms. Units for the autolysin activity were calculated from the same equation as given above.

RESULTS

Preliminary experiments

1) *Effect of some representative antibiotics on normal growth of strain NI-4.* Figure 1 shows the typical effect of some representative antibiotics on the normal growth of strain NI-4. Chloramphenicol, oxytetracycline, and erythromycin inhibited the cell growth at a dose of 200, 5, and 5 $\mu\text{g/ml}$, respectively. Rifampicin

Fig. 1. Effects of some representative antibiotics on normal growth of strain NI-4.

- no antibiotic (normal growth curve);
- ◆ 50 $\mu\text{g/ml}$ chloramphenicol (CP), 50 $\mu\text{g/ml}$ dihydrostreptomycin (DHSM);
- ▼ 0.5 $\mu\text{g/ml}$ mitomycin C (MC);
- ▽ 100 $\mu\text{g/ml}$ DHSM, 500 $\mu\text{g/ml}$ streptomycin;
- × 0.5 $\mu\text{g/ml}$ carzinophilin A (CZ A), 200 $\mu\text{g/ml}$ DHSM;
- △ 50 $\mu\text{g/ml}$ penicillin G, 500 $\mu\text{g/ml}$ cephaloridine, 500 $\mu\text{g/ml}$ DHSM;
- 5 $\mu\text{g/ml}$ oxytetracycline, 200 $\mu\text{g/ml}$ CP, 5 $\mu\text{g/ml}$ erythromycin;
- ◇ 1 $\mu\text{g/ml}$ rifampicin (RFP) and tolypomycin Y (TM Y), 10 $\mu\text{g/ml}$ MC;
- ▲ 5 $\mu\text{g/ml}$ RFP and TM Y, 5 $\mu\text{g/ml}$ CZ A, 1000 $\mu\text{g/ml}$ DHSM.

Antibiotics were added at early logarithmic phase ($\text{OD}_{660}=0.25$) to normal growing culture at the final concentrations indicated.

and tolypomycin Y inhibited the growth at 1 $\mu\text{g}/\text{ml}$, while, they produced lysis at above 5 $\mu\text{g}/\text{ml}$. Streptomycin inhibited the growth with a weak lysis at 500 $\mu\text{g}/\text{ml}$. Dihydrostreptomycin caused lysis at above 200 $\mu\text{g}/\text{ml}$. Carzinophilin A also had a lytic effect at above 0.5 $\mu\text{g}/\text{ml}$. No explanation is yet available as to the mechanism of the lytic action of rifampicin, tolypomycin Y, streptomycin, dihydrostreptomycin and carzinophilin A on the normal growing organisms. Penicillin G and cephaloridine revealed a typical lytic action. Mitomycin C induced a pronounced lysis with clostocin O production at a low concentration, whereas, at a high concentration, it caused growth inhibition without clostocin O production and cellular lysis.

2) *Lysis induction by mitomycin C treatment.* A typical induction pattern is shown in Fig. 2. The OD_{660} of culture increased for about 180 min after preincubation. Mature lysis began at about 180 min, and finished some 5 hr or more later (13). The lysis was caused by the breakdown of cell wall by clostocin

Fig. 2. Variation in lytic enzyme activities in mitomycin C-treated organisms of strain NI-4.

—○—, growth curve of mitomycin C-treated organisms; —△—, activity of autolysin; —●—, extracellular activity of clostocin O-endolysin; —●—, extracellular and intracellular activity of clostocin O-endolysin.

After mitomycin C treatment, the organisms harvested by centrifugation were re-suspended in a fresh medium prewarmed to 30° at the time indicated by a double arrow, and the incubation was continued at 30°. Assay of lytic enzyme activities is described in Method. Maximum activity of autolysin: 0.3 units. Maximum activity of clostocin O-endolysin: 12 units (on Formalin-treated organisms); 8 units (on cell wall).

O-endolysin. The development of the endolysin formation is also shown in Fig. 2. No extracellular activity of the endolysin could be detected until about 90 min after preincubation, but a small amount of active endolysin was present in the organisms at about 60 min. The content of endolysin increased with the age of the culture, a portion being released from the organisms. Maximum activity was observed about 30 min before the beginning of mature lysis. On the other hand, the content of active autolysin (originating from normal organisms) decreased with the age of the culture until about 30 min after preincubation. It was also found that the synthesis of autolysin was depressed by treatment with mitomycin C.

Effect of chloramphenicol on mitomycin C-induced lysis

1) *Effect of chloramphenicol added during induction of lysis of strain N1-4 by mitomycin C.* Aliquots of a culture were withdrawn at intervals to measure the effect of chloramphenicol on mitomycin C-treated organisms. After addition of chloramphenicol, the OD₆₆₀ was followed for 30 to 210 min. The results obtained from organisms harvested at different incubation period are shown in Fig. 3. Chloramphenicol inhibited both cell growth and mature lysis when added

Fig. 3. Effect of chloramphenicol added at various periods on mitomycin C-treated organisms of strain N1-4.

—○—, no CP; —▽—, at 0 min; —◇—, at 30 min; —◆—, at 60 min; —◇—, at 90 min; —△—, at 120 min; —▲—, at 150 min; —▴—, at 180 and 210 min.

After mitomycin C treatment, the organisms harvested by centrifugation were resuspended in a fresh medium prewarmed to 30° at the time indicated by a double arrow, and the incubation was continued at 30°. Chloramphenicol (CP, 50 µg/ml) was added at various periods indicated by the arrows, and incubation was continued for 30 min or more at 30°.

at any period up to 60 min after preincubation. However, when chloramphenicol was added at a later period when active endolysin was apparently present in the organisms (Fig. 2), premature lysis was observed. The rate of lysis increased with time, reaching its maximum after 180 min which was the time at which mature lysis was initiated. It seemed to be accelerated according to the increased concentration of active endolysin.

These results indicate that chloramphenicol provokes premature lysis in the presence of clostocin O-endolysin.

2) *Effect of concentration of chloramphenicol on premature lysis.* Experiments were then performed to study the effect of concentration of chloramphenicol on the premature lysis. Chloramphenicol of various concentrations was added at 150-min culture, in which organisms contained sufficient endolysin (Fig. 2). The premature lysis occurred after *ca.* 5-min lag period, and was completed by incubation for 60 min or more (Fig. 4). The rate of lysis was accelerated by the increased concentration of chloramphenicol. Chloramphenicol in a low concentration (5 to 50 $\mu\text{g/ml}$) to allow normal growth provoked premature lysis of mitomycin C-treated organisms.

Fig. 4. Effect of concentration of chloramphenicol on premature lysis.

—○—, no CP; —●—, 1 $\mu\text{g/ml}$ CP; —◇—, 5 $\mu\text{g/ml}$ CP; —▼—, 10 $\mu\text{g/ml}$ CP; —▽—, 25 $\mu\text{g/ml}$ CP; —▲—, 50 $\mu\text{g/ml}$ CP; —△—, 100 and 200 $\mu\text{g/ml}$ CP; —◆—, 500 $\mu\text{g/ml}$ CP.

Chloramphenicol (CP) was added at 150 min of culture. Other experimental conditions were the same as described in Fig. 3.

Table 2. Antibiotics behaving like chloramphenicol by provoking premature lysis.

Antibiotic	Effect on mitomycin C-treated organisms (Premature lysis) ^a μg/ml	Effect on normal growing organisms	
		(Static) ^c μg/ml	(Lytic) ^c μg/ml
Chloramphenicol	50	200	—
Thiophenicol	0.5	1	—
Mikamycin A	0.5	1	—
Mikamycin B	10	50	—
Chlortetracycline	0.5	1	—
Oxytetracycline	1	5	—
Erythromycin	1	10	—
Leucomycin	1	5	—
Oleandomycin	100	200	—
Spiramycin	10	50	—
Chromomycin A ₃	5	5	—
Mitomycin C	10	10	0.5 ^d
Actinomycin D	50 ^b	50	—
Actinomycin S	50 ^b	10	—
Antimycin A	200 ^b	200	—

^a Expressed as concentration of the antibiotic showing the same effect as that of 50 μg/ml chloramphenicol.

^b Expressed as concentration of the antibiotic showing the same effect as that of 10 or 25 μg/ml chloramphenicol.

^c Expressed as concentration of the antibiotic showing complete inhibition of cell growth or showing heavy cell lysis.

^d Mitomycin C-induced lysis.

— Cell lysis or inhibition of cell growth was not observed at above 500 μg/ml of the antibiotic.

Table 3. Antibiotics behaving like rifampicin or dihydrostreptomycin by provoking premature lysis.

Antibiotic	Effect on mitomycin C-treated organisms (Premature lysis) ^a μg/ml	Effect on normal growing organisms	
		(Static) ^c μg/ml	(Lytic) ^c μg/ml
Rifampicin	1	1	5
Tolypomycin Y	1	1	5
Dihydrostreptomycin	100	100	200
Bacitracin	1	50	100
Carzinophilin A	0.5	—	0.5
Streptomycin	500 ^b	500	1000
Novobiocin	100 ^b	100	500

^a Expressed as concentration of the antibiotic showing the same effect as that of 1 μg/ml rifampicin, or 100 μg/ml dihydrostreptomycin.

^b Expressed as concentration of the antibiotic showing the same effect as that of 0.5 μg/ml rifampicin, or 50 μg/ml dihydrostreptomycin.

^c Expressed as concentration of the antibiotic showing the complete inhibition of cell growth or showing heavy cell lysis.

— Cell lysis occurred even at 0.1 μg/ml.

Effect of many other antibiotics on mitomycin C-induced lysis

A systematic test using many antibiotics other than chloramphenicol was undertaken to determine whether any specific antibiotic might produce a premature lysis. Antibiotics shown in Tables 2 and 3 also inhibited both cell growth and mature lysis when added during the first 60 min after preincubation. However, when antibiotics were added later in the induction process, they provoked premature lysis as chloramphenicol did.

Many antibiotics shown in Table 2 behaved like chloramphenicol by provoking premature lysis. In all cases, premature lysis occurred after 5- to 7-min lag period, except some antibiotics. Chromomycin A₃, mitomycin C, actinomycins, and antimycin A took 10- to 15-min lag period. All antibiotics except mitomycin C caused growth inhibition without cellular lysis on normal growing organisms.

Fig. 5. Effect of rifampicin, toltypomycin Y, dihydrostreptomycin, bacitracin, and carzinophilin A on premature lysis.

A —○—, no antibiotic; —▼—, 0.5 µg/ml RFP and TM Y; —▲—, 1 µg/ml RFP and TM Y; —△—, 5 to 10 µg/ml RFP, 5 to 50 µg/ml TM Y; —◆—, 50 µg/ml RFP; ---▲---, 50 µg/ml CP.

B —○—, no antibiotic; —▼—, 10 µg/ml DHSM; —▲—, 50 µg/ml DHSM; —△—, 100 µg/ml DHSM, 1 µg/ml BC, 0.5 µg/ml CZ A; —◆—, 500 µg/ml DHSM; —◇—, 1000 µg/ml DHSM, 50 µg/ml BC, 5 µg/ml CZ A.

Rifampicin (RFP), toltypomycin Y (TM Y), dihydrostreptomycin (DHSM), bacitracin (BC), or carzinophilin A (CZ A) was added at 150 min of culture. Other experimental conditions were the same as given in Fig. 3. Deep color of rifampicin stopped the decrease in OD, in the case of its 50 µg/ml or more.

The antibiotics showing a much stronger inhibitory effect on normal growth were also effective on premature lysis.

The premature lysis provoked by rifampicin and tolypomycin Y occurred after 15- to 30-min lag period, and completed within about 60 min, as shown in Fig. 5. Rifampicin and tolypomycin Y took a longer lag period to the initiation of lysis than chloramphenicol did, but the rate of lysis displayed by them was similar to that by chloramphenicol, accompanied with rapid lysis. Dihydrostreptomycin, carzinophilin A, and bacitracin at a low concentration behaved like rifampicin and tolypomycin Y by provoking premature lysis, but, at a high concentration, they behaved like chloramphenicol (Fig. 4). Streptomycin and novobiocin behaved like dihydrostreptomycin by provoking premature lysis (Table 3). However, at above 500 $\mu\text{g/ml}$, they did not lyse mitomycin C-treated organisms at all. Streptomycin and novobiocin, as well as fradiomycin, kanamycin, polymyxin B, and viomycin, seemed to be chemical inhibitors of clostocin O-endolysin (18). These six antibiotics will be described in greater detail in the next paper. Rifampicin, tolypomycin Y, dihydrostreptomycin, bacitracin, car-

Fig. 6. Effects of oxytetracycline, chloramphenicol and erythromycin on mitomycin C-treated organisms of oxytetracycline-resistant mutant.

—○—, no antibiotic; —●—, 1 $\mu\text{g/ml}$ OTC; —▼—, 10 $\mu\text{g/ml}$ OTC; —▽—, 30 $\mu\text{g/ml}$ OTC; —△—, 100 $\mu\text{g/ml}$ OTC; —▲—, 50 $\mu\text{g/ml}$ CP, 1 $\mu\text{g/ml}$ EM; ---●---, 1 $\mu\text{g/ml}$ OTC on mitomycin C-treated organisms of wild strain.

Oxytetracycline (OTC), chloramphenicol (CP), or erythromycin (EM) was added at the time by the arrow, and incubation was continued for 180 min at 30°. The organisms used were resistant to 30 $\mu\text{g/ml}$ of OTC. Wild strain was also maintained as a control. Other experimental conditions and a symbol were the same as given in Fig. 3.

zinophilin A, streptomycin and novobiocin, which took a longer lag period to initiate premature lysis, produced lysis at appropriate concentration of them against normal growing organisms (Table 3).

Bleomycin, colistin, cycloheximide, lateriomycin, and sarcomycin were not effective against both mitomycin C-treated and normal growing organisms, even when above 500 $\mu\text{g/ml}$ of them were used.

Effect of oxytetracycline and others on mitomycin C-induced lysis of oxytetracycline-resistant mutant

To know in detail the action of antibiotics on premature lysis, an antibiotic-resistant mutant, oxytetracycline-resistant mutant (resistance to 30 $\mu\text{g/ml}$ oxytetracycline), was used as follows.

As shown in Fig. 6, oxytetracycline-resistant mutant was induced to lyse by mitomycin C treatment, as the wild strain. Oxytetracycline or erythromycin was added to a 150-min culture, in which the organisms contained sufficient endolysin. Mitomycin C-induced lysis of oxytetracycline-resistant mutant and its normal growth were hardly affected by less than 30 $\mu\text{g/ml}$ of oxytetracycline (being the permitted limit for the resistance), whereas both were sensitively affected by chloramphenicol, erythromycin, and 100 $\mu\text{g/ml}$ of oxytetracycline (being over the permitted limit for the resistance). In the case of wild strain, premature lysis was easily provoked by below 1 $\mu\text{g/ml}$ of oxytetracycline.

This result indicates that there is an antibiotic resistance in the premature lysis of mitomycin C-treated organisms. It is further clear that premature lysis may be due to the perturbation of biosynthesis in the organisms.

Effect of penicillin on mitomycin C-induced lysis

It is well known that penicillin inhibits the synthesis of cell wall peptidoglycan, and bacterial organisms are lysed by penicillin in the presence of autolysin without a concomitant inhibition of protein synthesis.

To know whether mitomycin C-treated organisms would be affected by penicillin G, 100 $\mu\text{g/ml}$ of penicillin G were added at intervals to mitomycin C-treated organisms. The lysis curves are shown in Fig. 7. Penicillin G evidently did not inhibit the synthesis of clostocin O-endolysin. There was a clear difference in the responses to penicillin G between normal growing and mitomycin C-treated organisms; the former, when exposed to penicillin G, took a 60-min lag period until initiation of lysis, while the latter harvested after a 30-min preincubation took a 90-min lag period. During this 90-min lag period, the activity of endolysin could hardly be detected, and the content of active autolysin became very small. Therefore, the temporal resistance of mitomycin C-treated organisms to penicillin G would depend on the decrease in the content of autolysin. However, the organisms returned to their state of high sensitivity after about 60 min. Their rate of lysis was greater than that of normal organisms. The lysis rate was accelerated still

Fig. 7. Effect of penicillin G added at various times on mitomycin C-treated organisms of strain NI-4.

—○—, no PC G; —▼—, at 0 min; —▽—, at 30 min; —◆—, at 60 min; —◇—, at 90 min; —△—, at 120 min; —▲—, at 150 min; —▲—, at 180 min; —▲—, at 210 min; ---○---PC G was added on normal growing organisms.

Penicillin G (PC G, 100 μ g/ml) was added at various periods indicated by the arrows, and incubation was continued for 30 min or more at 30°. Other experimental conditions and a symbol were the same as given in Fig. 3.

further by the increased content of endolysin (Fig. 2). The later penicillin G was added, the shorter was the lag period until the initiation of lysis. Similar results were obtained with cephaloridine and cephalosporin C, though their action was weaker than that of penicillin G.

These results indicate that mitomycin C-treated organisms continue the synthesis of cell wall which was inhibited by penicillin G. It is further shown that immediate inhibition of cell wall synthesis results in premature lysis in the presence of clostocin O-endolysin.

DISCUSSION

Mature lysis of mitomycin C-treated organisms of strain NI-4 failed when antibiotics such as chloramphenicol were added during the early part of the induction process. The inhibition of lysis depended on the almost immediate cessation of further synthesis of clostocin O-endolysin by the antibiotic treatment. However, premature lysis was provoked when antibiotics were added during a

late period when the endolysin had been synthesized. Similar phenomena are also known in the phage-infected organisms (19, 20). In our case, the relation of premature lysis to the synthesis of clostocin O-endolysin became clear, since each step of lytic process of mitomycin C-treated organisms was slow. The premature lysis certainly required the presence of clostocin O-endolysin whose enzymic properties will be reported in detail in a future paper.

Possible role of antibiotics in the premature lysis of mitomycin C-treated organisms might depend on two different actions of antibiotics; one depending on some perturbation of the metabolism of organisms and the other on their chelation for metallic ions which seem to protect the organisms from cellular lysis. We consider from the following observations that the action of antibiotics on the premature lysis might depend on the perturbation of cellular metabolism, especially that of protein synthesis: (1) Antibiotics showing a much stronger inhibitory effect on normal organisms were also effective against premature lysis; (2) protein synthesis inhibitors (such as chloramphenicol, thiophenicol, oxytetracycline, erythromycin, leucomycin, spiramycin, and mikamycins) responded more rapidly to the provocation of premature lysis than RNA synthesis inhibitors (such as actinomycins, rifampicin, and tolypomycin Y), DNA synthesis inhibitors (such as carzinophilin A, mitomycin C, and chromomycin A₃) and other type inhibitors (such as antimycin A) did; (3) antibiotic resistance was observed in the premature lysis system; (4) EDTA, sodium citrate, and 8-hydroxyquinoline in 10^{-2} to 10^{-3} M, which are the most effective chelating agent for metallic ions, had no effect on the premature lysis (unpublished data).

It is well known that penicillin and similar antibiotics inhibit the synthesis of cell wall peptidoglycan without a concomitant inhibition of protein synthesis, and bacterial organisms are lysed by penicillin in the presence of autolysin. It is further known that treatment of rapidly growing organisms with antibiotics, including chloramphenicol, mitomycin C, and tetracycline, results in rapid loss of their ability to autolyze and a decrease in their content of the active form of autolysin (21–24). Mitomycin C-treated organisms were lysed by penicillin G through the induction process. However, during the early part of the induction process, their lytic response to penicillin G treatment became temporarily delayed. The temporary resistance of mitomycin C-treated organisms to penicillin G could be explained on the same basis as the resistance to antibiotic-treated organisms in which active autolysin was very small. The high sensitivity of mitomycin C-treated organisms to penicillin at a later period would be due to the synthesis and increased content of clostocin O-endolysin. This observation indicates that mitomycin C-treated organisms continue the synthesis of cell wall peptidoglycan. It may be further said that the immediate inhibition of wall synthesis results in cellular lysis in the presence of clostocin O-endolysin. Bacitracin, dihydrostreptomycin, streptomycin, and novobiocin at a low concentration might behave like penicillin by provoking premature lysis, because these antibiotics are known

to inhibit cell wall synthesis or cytoplasmic membrane synthesis. On the other hand, since they are also known to inhibit protein synthesis, they might behave like chloramphenicol and others by provoking premature lysis or by inhibiting mature lysis. Since carzinophilin A, rifampicin, and tolypomycin Y also have a lytic effect on normal growing organisms, they might behave like penicillin by provoking premature lysis.

We speculate that the antibiotic-sensitive protein synthesis may be related to the continued synthesis of cell wall polymer. It may be further considered that the immediate block at some stage of protein synthesis by the antibiotics results in the inhibition of cell wall synthesis, and the immediate inhibition of cell wall synthesis results in premature lysis in the presence of clostocin O-endolysin.

We also would like to speculate on the governing factor in the lytic process of mitomycin C-treated organisms in addition to the action of endolysin as follows. Through the latent period of induction process, mitomycin C-treated organisms may continue a balanced growth with controlled cell wall hydrolysis by the endolysin and with cell wall synthesis. This controlled cell wall hydrolysis may facilitate insertion of newly synthesized wall materials into the new sites of cell wall synthesis. In the case of normal organisms, the controlled cell wall hydrolysis is known to be caused by autolysin (21, 25-27). At about 180 min after preincubation, the rate of cell wall hydrolysis may overcome the rate of cell wall synthesis. This unbalanced growth may be attributable to a decline in cell wall synthesis. Therefore, it may be considered that the mature lysis of mitomycin C-treated organisms is determined by the decline in cell wall synthesis in addition to the action of clostocin O-endolysin.

The authors thank Mr. S. Yoshino and Mr. K. Mori for their technical assistance in part of this work which was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education. Thanks are also due to Takeda Chemical Industries Co., Ltd., Kyowa Hakko Kogyo Co., Ltd., Kanegafuchi Chemical Industries Co., Ltd., and Eizai Co., Ltd., for their kind supply of the antibiotics.

REFERENCES

- 1) D. L. RALSTON, M. LIEBERMAN, B. BAER, and A. P. KRUEGER, *J. Gen. Physiol.*, **40**, 791 (1957).
- 2) D. L. RALSTON, B. BAER, M. LIEBERMAN, and A. P. KRUEGER, *J. Gen. Microbiol.*, **24**, 313 (1961).
- 3) G. KOCH and W. L. DREYER, *Virology*, **6**, 291 (1958).
- 4) T. KAGEYAMA, K. IKEDA, and F. EAGAMI, *J. Biochem. (Tokyo)*, **55**, 59 (1964).
- 5) C. C. DOUGHTY and J. A. MANN, *J. Bacteriol.*, **93**, 1089 (1967).
- 6) J. M. GOEFFERT and H. B. NAYLOR, *J. Virol.*, **1**, 701 (1967).
- 7) D. E. BRADLEY, *Bacteriol. Rev.*, **31**, 230 (1969).
- 8) N. B. GROMAN and G. SUZUKI, *J. Bacteriol.*, **86**, 187 (1963).
- 9) N. B. GROMAN, *J. Bacteriol.*, **90**, 1563 (1965).
- 10) A. W. HARRIS, D. W. A. MOUNT, C. R. FUERST, and L. SIMINOVITCH, *Virology*, **32**, 553

- (1967).
- 11) L. W. COHEN, *J. Virol.*, **4**, 209 (1969).
 - 12) L. W. COHEN, *J. Virol.*, **4**, 214 (1969).
 - 13) S. OGATA, O. MIHARA, Y. IKEDA, and M. HONGO, *Agr. Biol. Chem. (Tokyo)*, **36**, 1413 (1972).
 - 14) M. HONGO, H. ONO, K. KONO, and A. MURATA, *Agr. Biol. Chem. (Tokyo)*, **31**, 1070 (1967).
 - 15) S. OGATA and M. HONGO, *J. Gen. Appl. Microbiol.*, **19**, 251 (1973).
 - 16) M. HONGO, Y. TAHARA, and S. OGATA, *Agr. Biol. Chem. (Tokyo)*, **38**, 755 (1974).
 - 17) S. OGATA, Y. TAHARA, and M. HONGO, *Agr. Biol. Chem. (Tokyo)*, **38**, 763 (1974).
 - 18) S. OGATA and M. HONGO, *J. Gen. Microbiol.*, **18**, 315 (1974).
 - 19) J. TOMIZAWA and S. SUNAKAWA, *J. Gen. Physiol.*, **35**, 553 (1956).
 - 20) A. MURATA, E. SOEDA, and R. SARUNO, *J. Agr. Chem. Soc. Japan*, **25**, 32 (1970).
 - 21) G. D. SHOCKMAN, *Bacteriol. Rev.*, **29**, 345 (1965).
 - 22) M. L. HIGGINS, H. M. POOLEY, and G. D. SHOCKMAN, *J. Bacteriol.*, **103**, 504 (1970).
 - 23) H. M. POOLEY and G. D. SHOCKMAN, *J. Bacteriol.*, **103**, 457 (1970).
 - 24) C. R. STEWART and J. MARMUR, *J. Bacteriol.*, **101**, 449 (1970).
 - 25) J. S. ANDERSON, M. MATSUHASHI, H. A. HASKIN, and J. L. STROMINGER, *Proc. Natl. Acad. Sci. U.S.*, **53**, 881 (1965).
 - 26) F. E. YOUNG, *J. Biol. Chem.*, **241**, 3462 (1966).
 - 27) J. L. STROMINGER, K. IZAKI, M. MATSUHASHI, and D. J. TIPPER, *Fed. Proc.*, **26**, 9 (1967).