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Mode of Action of Clostocin O

Part 2. On the Synthesis of Nucleic Acid in Sensitive Bacteria

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An inducible bacteriocin clostocin O from *Clostridium saccharoperbutylacetonicum* (ATCC 13564) inhibited the both biosyntheses of DNA and RNA in sensitive organism. Especially, the biosynthesis of messenger RNA was most strongly affected and the biosynthesis of 23S ribosomal RNA was also affected by clostocin O infection. It also seemed that the release of 23S and 16S ribosomal RNA from the cellular membrane system easily occurred in clostocin O-infected organism.

INTRODUCTION

Clostocin O is a phage tail-like bacteriocin produced by *Clostridium saccharoperbutylacetonicum* (Ogata *et al.*, 1972), and inhibits the macromolecule biosynthesis of sensitive organism (Kato *et al.*, 1976) staying at the cell surface (Ogata *et al.*, 1976). But, it has been reported that some low molecular bacteriocins, such as colicins, can penetrate through the cell wall and cytoplasmic membrane into cytoplasm of sensitive organism.

Action of large molecular bacteriocin, such as clostocin O, may be explained by Nomura's hypothesis which suggests that there may be a specific relationship between bacteriocin particles and cytoplasmic membrane of sensitive organism and bacteriocin transmits its effect through a specific transmission system of cytoplasmic membrane to the final target in the infected organism (Nomura, 1964). It is very interesting theme to know the first target of action of large bacteriocin and transmission system.

As previously reported, clostocin O has a restricted receptor site (Ogata *et al.*, 1976) and inhibits the biosyntheses of macromolecules of infected organism, especially the biosynthesis of nucleic acids (Kato *et al.*, 1977). In this paper, we attempt to know the first target of clostocin O and to note the behavior of messenger RNA (m-RNA), because its turnover is the fastest in various nucleic acids.

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MATERIALS AND METHODS

Organisms

The bacterial strains used were N 1-4 (ATCC 13564) and No. 8 of *Clostridium saccharoperbutylacetonicum* for producing and sensitive strains of clostocin 0, respectively (Ogata et al., 1976).

Media and cultural conditions

The organisms were grown at 30°C under reduced atmospheric pressure (5 to 10 mmHg) in TYA medium (Ogata and Hongo, 1974) or CA-MM (Kato et al., 1976). TYA medium was used as preculture. To obtain a young exponentially growing culture, fresh CA-MM was inoculated with sufficient organism to produce an optical density (OD) of 0.15 at 660 nm, and unless otherwise mentioned the culture was incubated until its OD₆₆₀ became 0.3. Then, the culture was divided into 2 parts, and the incubation was followed at 30°C with bubbling N₂ gas. One part was added 30 units of clostocin 0, and the other was added heated clostocin 0 (100°C, for 10 min) for control. At 5 min after clostocin 0 infection ³H labeled compound was added both cultures, and a portion of cultures was taken out to fractionate the bacterial nucleic acid at suitable intervals.

Fractionation of RNA and DNA

The bacterial RNA and DNA were fractionated by modified STS procedure (Mizuno and Whitely, 1968).

Preparation of phenol-extractable RNA

Twenty ml of culture was taken out at suitable interval to extract with phenol containing sodium dodecyl sulfate (SDS) (Okamoto et al., 1962 ; Imamoto, 1969) and bentonite (Frankel-Conrat et al., 1961) to inhibit the activities of RNase and nuclease. Extracted RNA was precipitated by 95 % of ethyl alcohol (Forchhammer and Kjeldgaard, 1967; Mizuno et al., 1969). This procedure was repeated twice, as shown in Fig. 1.

Sucrose density gradient centrifugation of phenol-extractable RNA

The sucrose gradients were 5 to 20 % sucrose (Merck, Ltd.) in 0.02 M tris-HCl buffer containing 0.02 M NaCl. A 0.5 ml of phenol-extractable RNA solution was applied on the prepared gradients and was centrifuged at 23,000 rpm for 15 hr (Itoh et al., 1968; Muto, 1970) with SW 41 Ti rotor using ultracentrifuge (Beckman SPINCO, model L 3-50). Then, the solution was divided into 30 fractions (6 drops/tube) by the dropcounter (LKB 7000, ULTRORAC). Each fraction was measured the extinction at 260 nm using minicell by a photoelectric colorimeter (Spectrophotometer Hitachi 124).

Isotopes

³H-labeled uridine and thymidine were purchased from Daiichi Radioisotope Lab., Ltd..

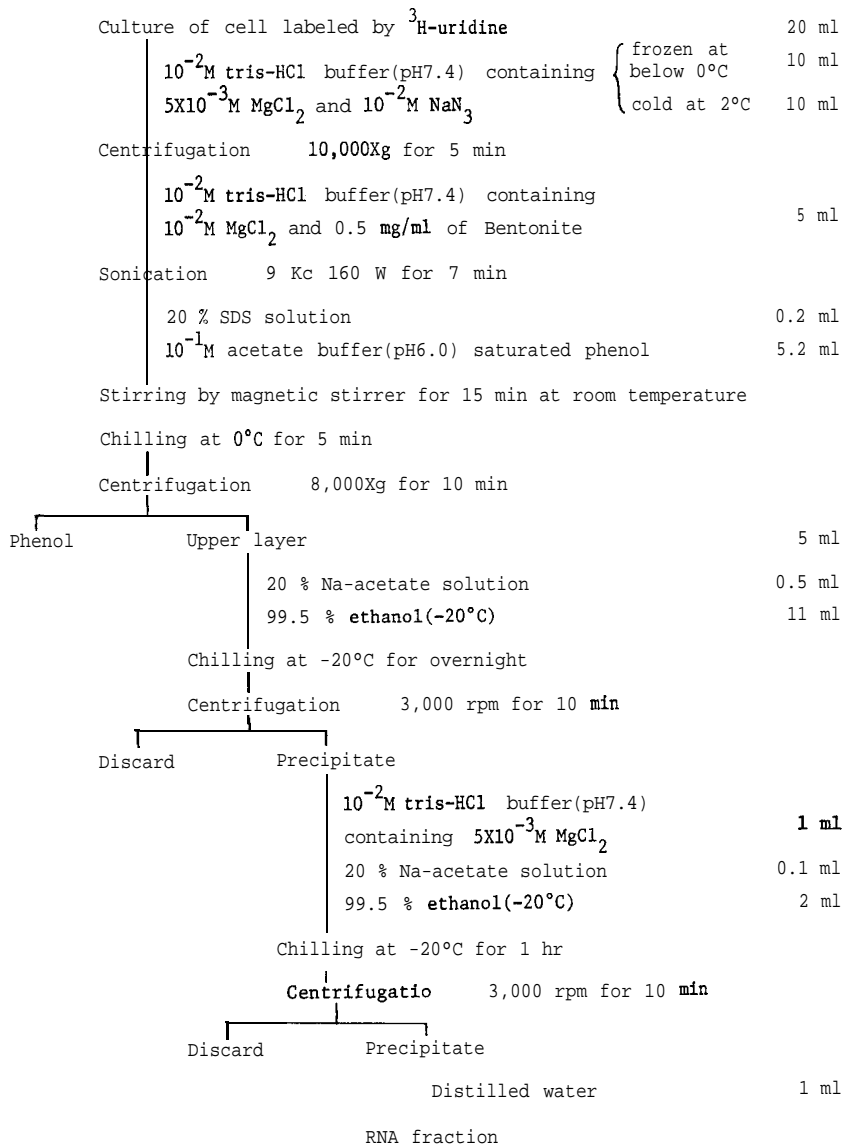


Fig. 1. Preparation of phenol-extractable RNA.

Measurement of radioactivity

Radioactivity was measured by a liquid scintillation counter (Beckman, model LS-250). The scintillation fluid consisted of 6 g of PPO (2,5-Diphenyl-oxazole, Beckman, Ltd.) in 500g of toluene.

RESULTS

Incorporation of ^3H into DNA fraction clostocin O-infected organism

The incorporation of ^3H -thymidine into DNA fraction was measured as shown in Fig. 2. It was observed that its incorporation into DNA fraction was strongly inhibited by clostocin O infection. This result agrees very closely with that obtained by the experiment using ^{32}P , described in the preceding paper (Kato *et al.*, 1978).

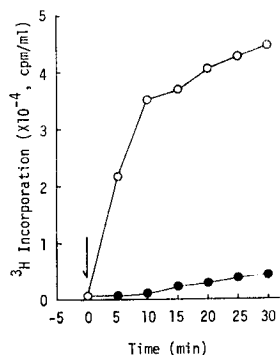


Fig. 2. Incorporation of ^3H -thymidine into DNA fraction. The sensitive organism was anaerobically incubated at 30°C with bubbling N_2 gas. At 5 min after infection of 30 units of clostocin O, ^3H -thymidine (final concentration of $0.5 \mu\text{Ci/ml}$) was added to the culture (shown by arrow). A portion of the culture was taken out at suitable intervals to fractionate with modified STS procedure. A 0.2 ml of the DNA fraction was measured radioactivity by a liquid scintillation counter. \circ , control (normal organism); \bullet , clostocin O-infected organism.

Incorporation of ^3H into RNA fraction of clostocin O-infected organism

The incorporation of ^3H -uridine into RNA fraction was measured at 1 min intervals. As shown in Fig. 3, the ^3H incorporation into RNA fraction of normal organism finished within 3 min after ^3H addition. On the other hand, ^3H incorporation into RNA fraction of clostocin O-infected organism was depressed in the early time after ^3H addition. This result indicates that clostocin O inhibits the incorporation of ^3H -uridine into the RNA which has the fastest turnover. When the labeling time is short, it is said that the almost all radioactivity would detect in m-RNA. To clear the inhibition of clostocin O on the biosynthesis of m-RNA, the incorporation of ^3H -uridine into phenol-extractable RNA will be measured in the next part.

Incorporation of ^3H -uridine into phenol-extractable RNA

As it is generally said that bacterial m-RNA is labeled by added isotope within a short time such as 1 to 2% of bacterial generation time. So, the sampling time was set up in short time, 30, 60, 120 and 240 sec after the addi-

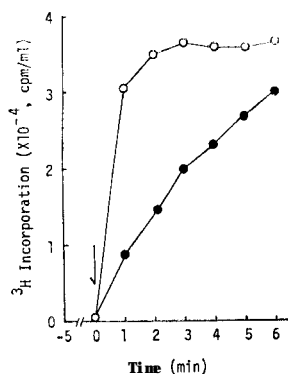


Fig. 3. Incorporation of ^3H -uridine into RNA fraction. Cultural and experimental conditions, and symbols are the same as described in Fig. 2. Arrow indicates the time of addition of ^3H -uridine (final concentration of $0.5 \mu\text{Ci/ml}$).

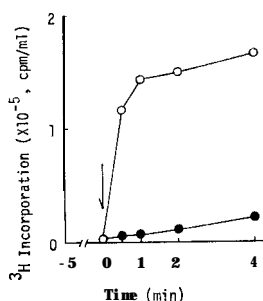


Fig. 4. Incorporation of ^3H -uridine into phenol-extractable RNA. Cultural condition and symbols are the same as described in Fig. 2. Arrow indicates the time of addition of ^3H -uridine (final concentration of $0.5 \mu\text{Ci/ml}$). Twenty ml of culture broth was used for the preparation of phenol-extractable RNA, as described in Fig. 1. A 0.1 ml of the RNA solution was measured radioactivity by a liquid scintillation counter.

tion of ^3H -uridine. RNA was extracted by phenol method as described in Materials and Methods, and precipitated with alcohol and then dissolved in 1 ml of distilled water. The incorporation into phenol-extractable RNA was strongly inhibited as shown in Fig. 4. It is clear that the main action of clostocin 0 toward RNA biosynthesis must occur m-RNA biosynthesis. The inhibition of incorporation of ^3H -uridine by clostocin 0 is stronger in phenol-extractable RNA than in RNA fraction of modified STS procedure. This result indicates that clostocin 0 may inhibit the polymerization of mononucleotides.

The extracted RNAs which were taken out at 30, 60 and 240 sec, were applied on the sucrose gradient centrifugation at 23,000 rpm for 15 hr, and then fractionated into 30 fractions. We measured the extinction at 260nm and the radioactivity of each fraction. As shown in Fig. 5, every sample had 3 peaks

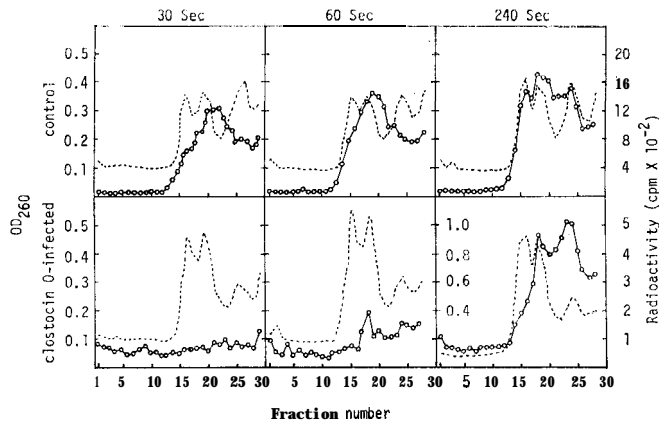


Fig. 5. Sucrose gradient sedimentation pattern of phenol-extractable RNA. The sample described in Fig. 4 was analysed by the sucrose density gradient sedimentation technique. A 0.5 ml of sample was layered on top of 11 ml sucrose gradient (5 to 20 %) containing 10^2 M tris-HCl buffer (pH 7.4) and 10^{-2} M NaCl. Centrifugation was performed at 23,000 rpm for 15 hr. The upper tier is the samples of control (normal organism), and the lower tier is the samples of clostocin O-infected organism. \cdots , OD_{260} ; \circ , radioactivity.

of OD_{260} , these peaks were considered as the RNA which had the sedimentation constant of 23S, 16S and 4S, from left to right in the figures, respectively (Gros et al., 1961). At 30 sec after the addition of 3H -uridine, there was no incorporation of radioactivity in the sample of clostocin O-infected organism, but in the case of control, the peak of radioactivity was observed at the position between 16S and 4S RNA. This peak represents m-RNA, and it is clear that the biosynthesis of m-RNA is inhibited by clostocin O infection. At 60 sec, the peak of m-RNA in control stayed at same position. In the case of clostocin O-infected organism, the incorporation of radioactivity into phenol-extractable RNA was hardly observed. At 240 sec, the peaks of radioactivity in control coincided in position with those of 23S, 16S and 4S RNA. In the case of clostocin O-infected organism, small peaks of radioactivity were observed at the positions of 16S and 4S RNA, but there was no peak of radioactivity at the position of 23S RNA; clostocin O inhibited the incorporation of 3H -uridine into 23S RNA. Also, the amounts of 16S and 23S RNA of clostocin O-infected organism were larger than of normal organism, so they may be liable to release from the cytoplasmic membrane and easily extracted as a result of infection of clostocin O.

DISCUSSION

Clostocin O affected the biosynthesis of nucleic acid in the sensitive organism. The incorporation of ^{32}P and 3H -thymidine into DNA fraction were strongly inhibited by clostocin O infection. The incorporation of ^{32}P into RNA

fraction was strongly inhibited, but that of ^3H -uridine was gradually increased in depressed rate as time passed. This difference may be caused by the kind of compound; inorganic phosphate and nucleoside. Figs. 4 and 5 indicate that clostocin 0 strongly inhibits the incorporation of ^3H -uridine into high molecular RNA; clostocin 0 inhibits the polymerization.

The remarkable effect of clostocin 0 was detected at first in the biosynthesis of m-RNA. Little is known about the effect of large bacteriocin on m-RNA. However, it is reported that ghosts of phage T4 inhibit immediately the biosynthesis of protein in sensitive organism, but does not affect on m-RNA (Fukuma and Kaji, 1972). So, it is specific for clostocin 0 to inhibit the biosynthesis of m-RNA. The causal relation between the inhibition of DNA biosynthesis and that of m-RNA biosynthesis is not clear at present time. But it is clear that the polymerization to high molecular nucleic acid, such as m-RNA and DNA, is specifically inhibited by clostocin 0 infection. Clostocin 0 may inhibit the biosynthesis of m-RNA as the first target and also inhibit polymerization of DNA and other RNA. Moreover, from the result in Fig. 5, we noticed that the ribosomal RNA released easily from the cytoplasmic membrane system as the secondary effect of clostocin 0 infection. The amounts of 23S and 16S RNA of clostocin O-infected organism were larger than that of uninfected organism, and increased with the passage of time. The both of 23S and 16S RNA of clostocin O-infected organism may be liable to be released from the cytoplasmic membrane, and they may be easily extracted.

Clostocin 0 inhibits the biosynthesis of m-RNA and DNA, staying at the surface of sensitive organism. Therefore, clostocin 0 may transmit its effect through a specific transmission system of cytoplasmic membrane to the final target in the infected organism. We are interested in the transmission system and wish to develop the study to elucidate the structure and function of biomembrane.

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