Mode of Action of Clostocin 0 : Part 1. On the Macromolecular Synthesis in Sensitive Bacteria

Kato, Fumio
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

Yoshino, Sadazo
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

Ogata, Seiya
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

Choi, Kyoung Ho
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

他

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Mode of Action of Clostocin 0

Part 1. On the Macromolecular Synthesis in Sensitive Bacteria

Fumio Kato*, Sadazo Yoshino, Seiya Ogata
Kyoung Ho Choi and Shinsaku Hayashida

Laboratory of Applied Microbiology, Faculty of Agriculture,
Kyushu University 46-02, Fukuoka 812

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Clostocin 0 is a phage tail-like bacteriocin produced by *Clostridium saccharoperbutylacetonicum* (ATCC 13564). One particle of clostocin 0 had an activity to kill one sensitive organism. The biosynthesis of macromolecules (protein, RNA and DNA) in sensitive organism was inhibited by clostocin 0 infection. The amounts of macromolecules of the infected organism were held at the initial level. The fate of the macromolecules in clostocin O-infected organism was investigated by using of isotope labeled compounds. The permeability for glucose or phenylalanine was held for 15 min after clostocin 0 infection. The biosyntheses of lipid and protein were somewhat moderately inhibited within 15 min after clostocin 0 infection, whereas the biosyntheses of DNA and RNA were immediately inhibited after clostocin 0 infection. However, degradation of DNA was not observed. From these results, we suggest that clostocin 0 strongly inhibits the synthesis of nucleic acids in short time.

INTRODUCTION

Bacteriocins are bactericidal substances produced by certain strains of bacteria and are usually active against the same or closely related species. Their narrow activity spectra and protein nature distinguish them from most of the other known antibiotics. They are conveniently divided into two groups (Reeves, 1972), one is comparatively small protein composed of low molecular weight under 100,000, and the other is made up of many protein components and shows phage tail-like structure.

The interest of bacteriocins in the molecular biology focuses mostly upon their characteristic mode of action. As yet, too few have been studied to generalize their mode of action. Bacteriocins, whose mode of action have made clear to date, are divided into 5 groups by their mode of action. The first group has colicin El and K (Jacob et ***al.***, 1952 ; Luria, 1964 ; Field and Luria, 1969), colicin A (Nagel de Zwaig, 1969) and colicin I (Levisohn ***et al.*** 1968) which inhibit energy metabolism. Namely, they inhibit the biosynthesis of macromolecules in consequence of the inhibition of oxidative phosphorylation. This group contains also phage tail-like bacteriocins, such as pyocin R (Kaziro and Tanaka, 1965) and pyocin 28 (Ohnishi, 1969). The second group

* Present address : Department of Agricultural Chemistry, Saga university, Saga

has colicin E2 (Obinata and Mizuno, 1970 ; Ringrose, 1970) and megacin C (Holland, 1963 ; Holland, 1965) which inhibit DNA synthesis following DNA degradation. Recently, colicin E2 has been reported to be a endonuclease (Schaller and Nomura, 1976). The third group has colicin E3 (Nomura, 1964) and cloacin DF 13 (de Graaf and Stouthamer, 1969) which inhibit protein synthesis. Bowman et *al.* (1971 a), Bowman et *al.* (1971 b) and Senir and Holland (1971) reported that colicin E3 may penetrate into the sensitive organism and directly act on the ribosome of infected organism as a sort of endonuclease. The fourth group has pneumocin G196 (de Graaf and Stouthamer, 1970) which inhibits both protein and RNA synthesis. The fifth group has megacin A-216 (Holland and Roberts, 1964) and enterococcin (Brock and Davie, 1963 ; Davie and Brock, 1966) which affect the cytoplasmic membrane of infected organism and inhibit the active transport and also cause a cellular lysis as a sort of phosphatase or phospholipase.

Clostocin 0 is a phage tail-like bacteriocin of *Clostridium sacchroperbutylacetonicum* (Ogata *et al.*, 1972) and one particle of clostocin 0 kills one sensitive organism after its adsorption to a specific receptor of the bacterial surface (Kato et *al.*, 1976 ; Ogata *et al.*, 1976). To make its mode of action clear, the metabolism of infected organism must be followed by using radioisotope labeled compounds.

This paper describes the effect of clostocin 0 on the biosynthesis of infected organism during short period after clostocin 0 infection. Further we attempt to know the mode of action of clostocin 0.

MATERIALS AND METHODS

Organisms

The strains used were N l-4 (ATCC 13564) and No. 8 of *Clostridium saccharoperbutylacetonicum* for producing and sensitive strain of clostocin 0, respectively.

Assay of clostocin 0

The activity of clostocin 0 was assayed by the double-layer method. Two fold serial dilutions of clostocin 0 were prepared, and examined by the spot test (Ogata *et al.*, 1972) on the sensitive organism. An arbitrary unit of activity (units/ml) was defined as the reciprocal of the highest dilution clearly showing an inhibition zone.

Assay of survival organism

The survival organisms infected by clostocin 0 were also assayed by double-layer method. The plate culture was incubated in the anaerobic jar for 24 hr and the colony number was counted by a colony counter (Kanto Rikaki Ltd.).

Labeled compounds

^{32}P labeled H_3PO_4 was used for measurement of nucleic acid synthesis. Phenylalanine-U-^{14}C was used for measurement of protein synthesis. Permeability and metabolism were investigated by using glucose-U-%. ^{32}P labeled

compound was purchased from the Japan Radioisotope Association and other compounds were purchased from Daiichi Radioisotope Lab. Ltd..

Media and cultural conditions

TYA medium (Ogata and Hongo, 1974) and minimum tnedium containing 2 g/l of Casamino acids (CA-MM) (Kato *et al. 1976)* were used for the culture broth of the sensitive organism. Glucose, Casamino acids and KH_2PO_4 was respectively cut down to one-tenth amount of CA-MM, when glucose-U-^{14}C, phenylalanine-U-^{14}C and ^{32}P were used. The sensitive organism preincubated in TYA medium was inoculated to 200 ml of CA-MM at 0.15 of OD_{660} and incubated until the OD_{660} reached at 0.3 under reduced atmospheric pressure (5 to 10 mmHg). This culture broth was divided into equal volume of 90 ml and continued incubation bubbling with high purity of N_2 gas at 30°C. A 10 ml of 300 units of clostocin 0 was added to one culture and heated clostocin 0 (inactivated clostocin 0) was added to control culture. After 5 min, radioisotope compound was added to both cultures (0 min). Then 4.5 ml of culture broth was taken out at suitable intervals and the macromolecules of the organisms were fractionated by modified Schmidt-Thannhauser-Schneider (STS) procedure (Mizuno and Whitely, 1968). The production of clostocin 0 had been described in elsewhere (Ogata *et al.,* 1972).

Fractionation of cellular components

The fractionation of macromolecules and other cellular components were performed by modified STS procedure. A 0.5 ml of 50 % (w/v) trichloroacetic acid (TCA) was immediately added to 4.5 ml of cell culture to stop the incorporation of radioisotopes. When further experiments were performed on this fraction, the organism was centrifuged immediately under cold, and then treated with cold TCA.

The fractionation of nucleotide and sugar phosphate ester was attempted from the cold acid soluble fraction by using treatment of activated charcoal (Norit A) reported by Leloir and Cardini (1957).

Estimation of the amounts of some cellular components labeled by ^{32}P

The sensitive organism preincubated in TYA medium was inoculated at 0.15 of OD_{660} in CA-MM reducing KH_2PO_4, then ^{32}P was added, and incubated at 30°C under reduced atmospheric pressure until OD_{660} became to be at 0.3. The sensitive organism was harvested by centrifugation at 10, $000 \times g$ for 15 min, and resuspended in fresh 200ml of CA-MM, then the culture was divided into two parts of 90 ml. The cultural condition and fractionation was performed as same as above mentioned.

Measurement of radioactivity

The radioactivity of ^{14}C and ^{32}P was counted by Aloka gas flow counter (model TDC-10).

RESULTS

Single hit killing kinetics of clostocin 0

Various concentrations of clostocin 0 were infected to the sensitive or-

ganism at 30°C for 15 min, and the survival numbers were detected by the double-layer method. As shown in Fig. 1, the concentration of clostocin 0 and logarithm of the survival ratio bears a liner relationship to each other. This result must indicate that the killing ability of clostocin 0 complies with a single hit theory. According to this single hit theory, one killing unit kills 36% of the organisms. From the result in Fig. 1, we are able to know that 3 units of clostocin 0 can kill 36% of the organisms. Three units of clostocin 0 are equal to 3x 10^8 particles/ml (Ogata *et al.,* 1976). In the experiment of Fig. 1, we used 2×10^8 cells/ml of the sensitive organism. Therefore, 1.5 particles of clostocin 0 kill one organism. This fact indicates that the killing kinetics of clostocin 0 follows the single hit theory.

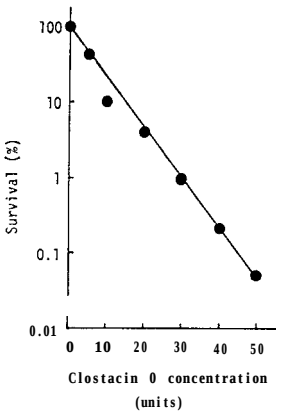

Fig. 1. Dependence of various concentrations of clostocin O on its killing activity. Various concentrations of clostocin O were infected to the sensitive organisms at 30°C for 15 min. Survivors were counted by double-layer method.

Inhibition of macromolecule biosynthesis by clostocin 0

To know the influences of clostocin 0 on the biosyntheses of macromolecules in the sensitive organism, the amounts of the macromolecules were measured after clostocin 0 infection. The dose of clostocin 0 used for this experiment was enough to kill 95 % or more of the sensitive organism which was grown in CA MM. As shown in Fig. 2, the amounts of protein, RNA and DNA were all kept at the initial level and did not change for 3 hr. This result indicates that the biosyntheses of these macromolecules are inhibited by clostocin 0 infection, and moreover, these substances are not degraded by clostocin 0 infection.

Permeability of clostocin O-infected organism

The uptake of ^{14}C-glucose or ^{14}C-phenylalanineby the clostocin O-infected organism was measured at intervals of 5 min after addition of radioisotopes. Results are shown in Fig. 3. The uptake of ^{14}C-glucose was not inhibited during early 10 min and the uptake of ^{14}C-phenylalanine also continued for 15 min under reduced rate. These results indicate that the permeability of in-

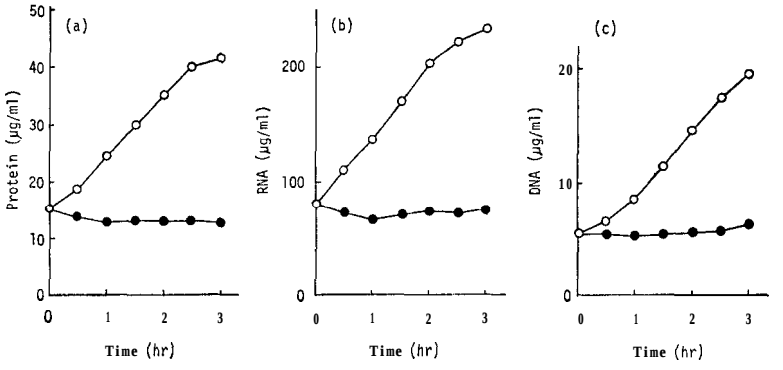

Fig. 2. Effects of clostocin 0 on macromolecules biosynthesis of infected organism. Sensitive organism was incubated in the minimum medium containing casamino acids and added 39 units of clostocin 0 at 0.3 of OD_{660}. Ten ml of the culture broth was taken out at each 30 min intervals and fractionated by modified STS procedure. (a) protein, (b) RNA, (c) DNA. ○, control (normal organism) ; ● , clostocin O-infected organism.

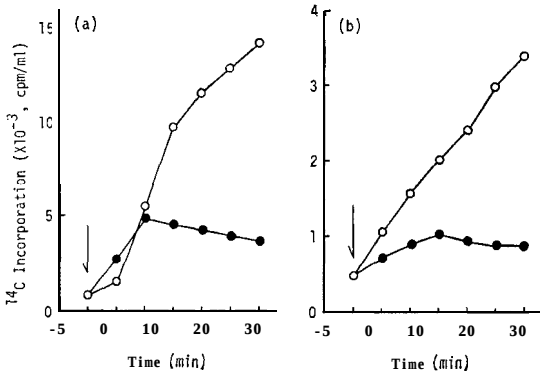

Fig. 3. Uptake of ^{14}C-glucose and ^{14}C-phenylalanine by clostocin O-infected organism. The organism was incubated at 30°C with bubbling N_2 gas. At 5 min after infection of 30 units of clostocin 0, radioisotope was added to the culture, as shown by the arrow. A 4.5 ml of the culture was taken out at 5 min interval and chilled at each time. The organism was harvested by centrifugation at 3,000 rpm for 10 min and was degradated with 0. 3 N KOH. An amount of 0. 5 ml of sample was dried up in a planchette and radioactivity was measured by a gas flow counter. (a) uptake of ^{14}C-glucose (final concentration of 0. 5 μCi/ml), (b) uptake of ^{14}C-phenylalanine (final concentration of 0.25 &i/ml). ○, control (normal organism) ; ● , clostocin O-infected organism.

fected organism is not affected by clostocin 0 in the early stage of infection, and that the inhibition of macromolecule synthesis is not due to the decrease in permeability.

Macromolecular synthesis in clostocin O-infected organism

'The incorporation of radioisotopes into lipid, protein, RNA and DNA frac-

tions was measured. As shown in Fig. 4 (a), ^{32}P incorporation into lipid fraction did not show a great difference between control and clostocin O-infected organism during early 15 min. Incorporation of ^{14}C-phenylalanine into protein fraction continued for 20 min in a reduced rate [Fig. 4 (b)]. However, as shown in Fig. 4 (c) and (d), the incorporation of ^{32}P into RNA and DNA fractions was strongly inhibited in the infected organism. These results indicate that clostocin 0 inhibits abruptly the synthesis of nucleic acid in the infected organism.

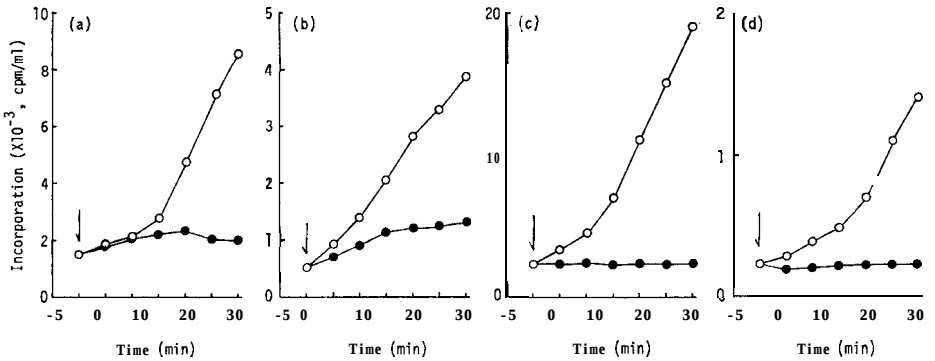

Fig. 4. Incorporation of radioisotopes into lipid, protein, RNA and DNA fractions after clostocin 0 infection. The cultural condition and symbols are the same as described in Fig. 3. The arrow indicates the time of addtion of radioisotope. An amount of 4.5 ml of the culture was withdrawn at 5 min interval and fractionated by the modified STS procedure. (a) Incorporation of ^{32}P into lipid fraction (final concentration of ^{32}P-H_3PO_4 was 2 &i/ml), (b) incorporation of ^{14}C-phenylalanine into protein fraction (final concentration of ^{14}C-phenylalanine was 0.25 μCi/ml), (c) incorporation of ^{32}P into RNA fraction (final concentration of ^{32}P-H_3PO_4 was 2 μCi/ml), (d) incorporation of ^{32}P into DNA fraction (final concentration of ^{32}P-H_3PO_4 was 2 μCi/ml).

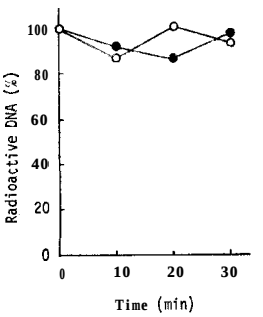

Fig. 5. Fate of ^{32}P-labeled DNA after clostocin 0 infection. The sensitive organism grown in CA-MM with ^{32}P-H_3PO_4 (final concentration of 2.5 μCi/ml) was harvested by centrifugation and resuspended in the fresh CA-MM without ^{32}P. After infection of clostocin 0, a portion of culture was withdrawn at 10 min interval and fractionated by modified STS procedure. Symbols are the same as described in Fig. 3.

Fate of DNA in clostocin O-infected organism

To determine whether clostocin 0 causes the degradation of DNA or not, following experiment was performed. Clostocin 0 was infected to ^{32}P labeled organism and the amounts of radioactive DNA were measured. As shown in Fig. 5, the amounts of radioactive DNA were almost the same in infected and uninfected organisms. So the significant degradation of DNA did not occur for 30 min after clostocin 0 infection. It is concluded that clostocin 0 inhibits the biosynthesis of nucleic acid but does not have the activity to degradate DNA like colicin E2 (Obinata and Mizuno, 1970).

Biosynthesis and degradation of organic phosphate compounds in clostocin O-infected organism

Clostocin 0 strongly inhibited the nucleic acid of sensitive organism. So it was considered that the effects of clostocin 0 on the biosynthesis and degradation of organic phosphate compounds had to be investigated as the next step. These substances can be detected in cold TCA soluble fraction as ^{32}P

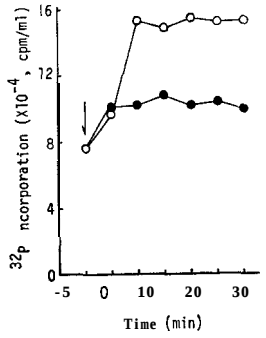

Fig. *6.* Incorporation of ^{32}P into cold TCA soluble fraction after clostocin 0 infection. Experimental conditions and symbols are the same as described in Fig. 3.

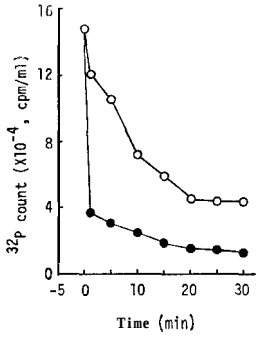

Fig. 7. Decrease of cold TCA soluble substances labeled with ^{32}P after clostocin 0 infection. Experimental conditions and symbols *are* the same as described in Fig. 3.

compounds when ^{32}P was used. The changes of radioactivity between control and the infected organism were monitored in this fraction. The incorporation of ^{32}P into clostocin O-infected organism is shown in Fig. 6. And the decrease of radioactivity in the organism which had been labeled with ^{32}P is shown in Fig. 7. The incorporation of ^{32}P was repressed by clostocin 0 infection. The radioactivity of labeled organism was suddenly dropped by clostocin 0 infection.

As the cold TCA soluble fraction was considered to contain nucleotides and other phosphate compounds, mainly sugar phosphate esters, these compounds were separated with activated charcoal. The nucleotides were adsorbed to the activated charcoal, on the other hand, the sugar phosphate esters and others were not adsorbed: the radioactivities of each part were measured

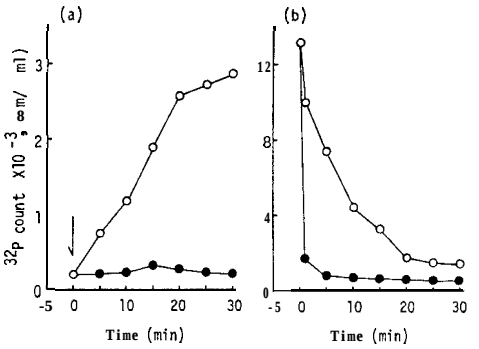

Fig. 8. Change in nucleotidc level after clostocin O infection. A 0.5 ml of cold TCA soluble fraction was treated with activated charcoal and the charcoal-adsorbed fraction was measured its radioactivity by gas flow counter. (a) incorporation of ^{32}P labeled nucleotide, (b) decrease of ^{32}P labeled nucleotide. Symbols are the same as described in Fig. 3.

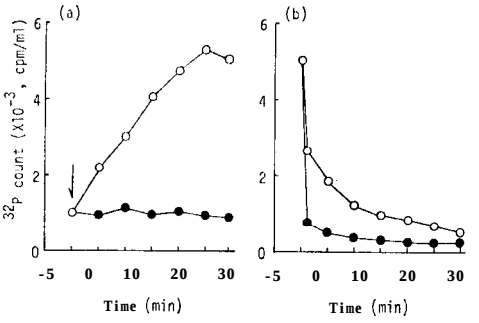

Fig. 9. Change in level of radioactivity in activated charcoal-unadsorbed fraction after clostocin 0 infection. A 0.5 ml of cold TCA soluble fraction was treated with activated charcoal and the unadsorbed-fraction was measured its radioactivity by gas flow counter. (a) incorporation of ^{32}P into activated charcoal-unadsorbed fraction, (b) decrease of ^{32}P labeled substances. Symbols are the same as described in Fig. 3.

as shown in Figs. 8 and 9. As shown in Fig. 8 (a), the incorporation of ^{32}P into nucleotides was remarkably inhibited by clostocin 0 infection. Also, Fig. 8 (b) indicates that the decrease of labeled nucleotides remarkably occurred within a short period by clostocin 0 infection. It is considered that the strong inhibition of nucleotide biosynthesis and the remakable release of nucleotide from clostocin O-infected organism may cause the inhibition of nucleic acid biosynthesis system. The changes of radioactivity in the substances unadsorbed by activated charcoal is shown in Fig. 9 (a). The incorporation of ^{32}P into sugar phosphate esters was strongly inhibited by clostocin 0 infection. And also the decrease of labeled substances in infected organism was larger than that of control as shown in Fig. 9 (b). Thus, it seems that the biosynthesis of sugar phosphate esters is strongly inhibited and the release of these substances from the infected organism is hastened by clostocin 0 infection.

DISCUSSION

The biosynthesis of nucleic acid of clostocin O-infected organism was strongly inhibited. But, their permeability and biosynthetic abilities of lipid and protein remained for 15 to 20 min after clostocin 0 infection. So it was considered that clostocin 0 acted upon the biosynthesis of nucleic acid as the first target. The mode of action of clostocin 0 was considered to be closely related to those of colicins El and K, and pyocins R and 28, and also that of colicin E 2. However, there are some differences in mode of action between clostocin 0 and these bacteriocins. Pyocin R inhibits the biosynthesis of protein (Kaziro and Tanaka, 1965) and pyocin 28 inhibits strongly the biosynthesis of almost all macromolecules at the same grade (Ohnishi, 1969). Colicins E 1 and K inhibit such all abilities of sensitive organism as the biosynthesis of macromolecules, permeability and biosynthesis of ATP, but the respiratory ability is retained for considerable time (Nomura, 1964). Colicin E 2 causes remarkable DNA degradation [Holland and Holland, 1970; Yanai et *al.*, 1973), and moreover, RNA and protein of 50 S and 30 S ribosomes of sensitive organism are degradated into low molecules (Nose and Mizuno, 1968; Nose *et al.*, 1966). From these facts, it is concluded that clostocin 0 inhibits only biosynthesis of nucleic acid and shows a different type of mode of action from these bacteriocins.

Duckworth (1970), Fukuma and Kaji (1972) and Winkler and Duckworth (1971) reported that phage ghosts had shown the same action as bacteriocins. The ghosts of T4 phage cause not only the repression of permeability and the inhibition of biosynthesis of macromolecules but also remarkable inhibition of respiratory ability in infected organism. As the permeability and protein biosynthesis are strongly decreased at first by the infection of phage ghosts, it seems that their mode of action is not the same as that of clostocin 0.

The biosyntheses of nucleotides in cold TCA soluble was strongly inhibited by clostocin 0 infection, so this fact was considered to be closely related to the inhibition of nucleic acid biosynthesis and energy metabolism in clostocin O-infected organism.

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