Killing Activity of Clostocin O[†]

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Clostocin O is a phage tail-like bacteriocin produced by *Clostridium saccharoperbutyl-acetonicum* NI-4. One particle of clostocin O had an activity to kill one sensitive organism. Clostocin O had also the lytic activity, but this lytic activity was not an essential action of clostocin O, because clostocin O was able to show a sufficient killing activity even under the condition to inhibit its lytic activity. The biosynthesis of macromolecules (protein, RNA and DNA) in sensitive organisms was inhibited by clostocin O infection. The amounts of macromolecules of the infected organisms were held at the initial level.

Clostocin O is a large bacteriocin produced by *C. saccharoperbutylacetonicum* NI-4 and has a characteristic to show a remarkable lytic activity against the sensitive organisms.¹⁾ Among the phage tail-like bacteriocins including clostocin O, only pyocin R^{2,3)} and pyocin 28⁴⁾ have been studied to reveal the mode of actions in detail but those of other bacteriocins have not been studied in detail yet. These bacteriocins are distinctive to have higher killing rate than small bacteriocins. For example, it has been reported that one killing unit of colicin E2-P9 or E3-CA38 corresponds to 40~300 molecules,⁵⁾ but that of large bacteriocin corresponds to one particle.⁶⁾

In general, the killing activity of bacteriocin can be detected by the measurement of colony forming ability of infected sensitive organisms, but in the case of clostocin O, which has a remarkable lytic activity, the killing activity must be measured under the condition to inhibit the cellular lysis.

This paper describes the killing rate and the relation between lytic activity and killing activity of clostocin O, and the effect on the biosynthesis of macromolecules in the infected organisms.

MATERIALS AND METHODS

Organisms. The strains used were NI-4 (ATCC Bacteriocins of Nonpathogenic *Clostridium* Species. Part VI.

13564) and No. 8 of *Clostridium saccharoperbutylacetonicum* for producing and sensitive strain of clostocin O, respectively.⁷⁾

Media and cultural conditions. The organisms were grown at 30°C under reduced atmospheric pressure (5 to 10 mmHg) in TYA medium⁷⁾ or minimum medium (MM) which contained (g/liter distilled water): glucose, 20; ammonium acetate, 1; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.01; biotin, 10⁻⁴; at pH 6.5. CA-MM was also used, supplemented 2 g/liter of casamino acids to the MM. To obtain a young exponentially growing culture, fresh medium was inoculated with sufficient organisms to produce an initial optical density (O. D.) of 0.1 at 660 nm, and unless otherwise mentioned the culture was incubated until its O. D.₆₆₀ became 0.3 (ca. 1 to 2 × 10⁸ cells/ml).

Turbidity measurement of bacterial culture. Optical density of bacterial culture was measured at 660 nm in a photoelectric colorimeter (model 7A, Tokyo Kodens Ltd.) as previously described.⁸⁾

Assay of clostocin O. The activity of clostocin O was assayed by the double-layer method. Two fold serial dilutions of clostocin O were prepared, and examined by the spot test⁷⁾ on the sensitive organisms. An arbitrary unit of activity (units/ml) was defined as the reciprocal of the highest dilution clearly showing an inhibition zone.

Assay of survival organisms. The survival organisms infected by clostocin O 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.).

Inhibition of lytic activity of clostocin O by fradiomycin. Fradiomycin was used to inhibit the lytic

activity of clostocin O.⁸⁻¹¹⁾ A 100 to 500 $\mu\text{g/ml}$ of fradiomycin was added to the culture of sensitive organism at the same time of clostocin O addition.

Analysis of macromolecules in the sensitive organisms. The sensitive organisms were inoculated to 100 ml of CA-MM to produce an initial O. D. of 0.1 at 660 nm, and incubated at 30°C under a reduced atmospheric pressure. When the O. D.₆₆₀ of the culture became 0.3, 30 units of clostocin O was added to the culture and for control heated clostocin O (100°C, for 10 min) was added. A 10 ml of the culture broth was taken out at each 30 min intervals to fractionate the macromolecules by modified Schmidt-Thannhauser-Schneider (STS) procedure for RNA, DNA and protein. RNA was measured by orcinol reaction,¹²⁾ DNA by Burton's modification,¹³⁾ and protein by the method of Lowry *et al.*,¹⁴⁾ respectively.

RESULTS

Selection of the condition inhibiting the lytic activity of clostocin O

Clostocin O easily induced the cellular lysis of sensitive organisms. Therefore, to distinguish between lytic and killing activity, the condition was examined, in which only the former activity of clostocin O was inhibited. Thirty units of clostocin O was infected to the sensitive organisms grown in various media until their O. D.₆₆₀ were 0.3. As shown in Fig. 1, a remarkable lysis of the organisms grown in TYA medium occurred, but in the case of

minimum medium containing casamino acids (CA-MM) the lysis was strongly inhibited. Moreover, when 100 $\mu\text{g/ml}$ of fradiomycin was added to CA-MM, the lysis was almost completely inhibited.

Killing rate and adsorption rate using the minimum medium

The clostocin O-induced lysis was inhibited when the organisms grown in the minimum medium were used. To ascertain the cause of this lysis inhibition, the killing rate and adsorption rate of clostocin O were measured using the organisms grown in the CA-MM. The survivor was 1% using TYA medium and 4% using CA-MM when 30 units of clostocin O was infected to the sensitive organisms for 15 min, as shown in Table I. As shown in Table II, the adsorption rate was 90% using TYA medium, but 30% using CA-MM. From these results, it appears that the sensitive organisms grown in the CA-MM have fewer receptors than the organisms grown in TYA medium. It is also said that excess clostocin O can not be adsorbed to the organisms grown in the CA-MM and then the cellular lysis hardly occurs.⁷⁾ The killing rate, however, was not different using each medium. Therefore, it is proved that it is favorable to use the minimum medium in determining the effect

FIG. 1. Clostocin O-induced Lysis of the Sensitive Organisms Grown in Various Media.

Thirty units of clostocin O were infected to the sensitive organisms grown in (a) TYA medium, (b) minimum medium containing casamino acids, or (c) minimum medium containing casamino acids and 100 $\mu\text{g/ml}$ of fradiomycin. Initial O. D.₆₆₀ was 0.3, which was expressed as 100%. ○—○, control, no addition of clostocin O; ●—●, addition of 30 units of clostocin O.

for the macromolecules biosynthesis of the sensitive organisms by clostocin O.

Single hit killing kinetics of clostocin O

Various concentrations of clostocin O were infected to the sensitive organisms at 30°C for 15 min, and the survival numbers were detected by the double-layer method. As shown in Fig. 2, the concentration of clostocin O and logarithm of the survival ratio bears a liner relationship to each other. This result should indicate that the killing ability of clostocin O 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. 2, we are able to know that 3

FIG. 2. 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.

units of clostocin O can kill 36% of the organisms used. Three units of clostocin O are equal to 3×10^8 particles/ml.⁷⁾ In the experiment of Fig. 2, we used 2×10^8 cells/ml of the sensitive organism. Therefore 1.5 particles of clostocin O kill one organism. This fact indicates that the killing kinetics of clostocin O follows the single hit theory.

TABLE I. SURVIVAL NUMBERS OF CLOSTOCIN O-INFECTED ORGANISMS USING TYA AND MINIMUM MEDIUM

Sensitive organisms grown in TYA medium or minimum medium containing casamino acids (CA-MM), were infected by 30 units of clostocin O at 30°C for 15 min, and then the survivors were measured by double-layer method.

Medium	Dose of clostocin (units)	Survival cell number (cells/ml)	Survival (%)
TYA	0	2×10^8	100
	30	2×10^6	1
CA-MM	0	1.5×10^8	100
	30	6×10^8	4

TABLE II. ADSORPTION OF CLOSTOCIN O TO THE SENSITIVE ORGANISMS GROWN IN TYA AND MINIMUM MEDIUM

Sensitive organisms grown in TYA medium or minimum medium containing casamino acids (CA-MM) were infected by 30 units of clostocin O at 30°C for 30 min. The cultures were centrifuged at $10,000 \times g$ for 10 min and the supernatants were assayed for residual clostocin O activities by spot test.

Medium	Dose of clostocin (units)	Unadsorbed clostocin (units)	Adsorbed clostocin (%)
TYA	30	2	93
CA-MM	30	20	33

Fate of clostocin O-infected organisms in the presence of fradiomycin

In the previous papers,⁸⁻¹¹⁾ we reported that fradiomycin was a strong inhibitor toward some lytic enzymes. Fradiomycin could strongly inhibit clostocin O-induced lysis. So the killing rate of clostocin O-infected organisms was measured in the presence of fradiomycin by means of counting the survivors, as shown in Table III. The normal organisms were not affected at all by the treatment of 500 $\mu\text{g/ml}$ of fradiomycin for 15 min. The survivors of clostocin O-infected organisms were 0.5 to 1% in spite of existence or absence of fradiomycin. These results indicate clearly that the killing ability of clostocin O is not reduced by the inhibition of the cellular lysis. But it is unfavorable to use fradiomycin in the

TABLE III. EFFECT OF FRADIOMYCIN ON THE KILLING ACTIVITY OF CLOSTOCIN O

Thirty units of clostocin O were infected to the sensitive organisms at 30°C for 15 min, and survivors were measured by double-layer method.

Addition	Cell number (cells/ml)	Survival (%)
No addition	1.5×10^8	100
Fradiomycin (500 $\mu\text{g/ml}$)	1.6×10^8	100
Clostocin O (30 units)	1.5×10^6	1.0
Fradiomycin (500 $\mu\text{g/ml}$)		
+	7.6×10^5	0.5
Clostocin O (30 units)		

experiment to determine the effects for the biosynthesis of macromolecules in the sensitive organisms by clostocin O, because fradiomycin is a strong inhibitor of protein biosynthesis.

Inhibition of macromolecule biosynthesis by clostocin O

To know the influences of clostocin O on the biosynthesis of macromolecules in the sensitive organisms, the amounts of their macromolecules were measured after clostocin O infection. The dose of clostocin O used for this experiment was enough to kill 95% or more of the sensitive organisms which were grown in CA-MM. As shown in Fig. 3, 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 biosynthesis of these

FIG. 3. Effects of Clostocin O on Macromolecules Biosynthesis of Infected Organisms.

Sensitive organisms were incubated in the minimum medium containing casamino acids and added 30 units of clostocin O at 0.3 of O. D.₈₀₀. A 10 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 organisms; ●—●, clostocin O-infected organisms.

macromolecules are inhibited by clostocin O infection, and moreover, these substances are not degraded by clostocin O infection.

DISCUSSION

Clostocin O had an ability to kill one sensitive organism by one particle similarly to other phage tail-like bacteriocins and phage ghosts. The decrease of optical density of the infected culture was observed within 10 min after clostocin O addition when the usual TYA

medium was used. On the other hand, this cellular lysis was inhibited by fradiomycin or using minimum medium. However, the killing activity of clostocin O was not affected by using fradiomycin. Therefore, it is said that lytic activity of clostocin O is due to the lytic enzyme¹⁾ attached to clostocin O particles, and the lytic activity of clostocin O is not an essential action of clostocin O.

When clostocin O infected the sensitive organisms, the biosynthesis of their macromolecules (protein, RNA and DNA) was all inhibited, but the degradation of these substances was not observed. Clostocin O seemed to kill the sensitive organisms by the inhibition of the macromolecule biosynthesis. To elucidate the mode of action of clostocin O, it must be clarified where is the first point of the inhibition in the macromolecules biosynthesis of the infected organisms. For this purpose, the experiment must be done with the radioisotope labeled compounds to determine the effect of clostocin O on the incorporations of radioactivity into protein, nucleic acid and other fractions in a short time after clostocin O infection. The results will be described in forthcoming papers of this series.

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