Rod-shaped Pyocin 28

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SUMMARY

Pyocin 28 was obtained by induction of Pseudomonas aeruginosa P28 with mitomycin. Pyocin activity was correlated with the number of rod-shaped particles in purified preparations. The width of the pyocin rod was uniform, measuring about 90 Å, but the length was not uniform, varying from 200 to 4000 Å, but rods measuring 1000 to 1200 Å were most frequent. A dark central line and regular cross-striations were usually seen on the rod, and a fine fibre was sometimes visible at the sharp end. Pyocin activity was slightly reduced from ultrasonic treatment, but not at all by trypsin, Nagarse, DNase and RNase. The pyocin was stable between pH 5.0 and 8.0, and was completely inactivated by heating at 60° for 10 min. Specific attachment of numerous pyocin rods to the surface of sensitive bacteria was observed by the electron microscope.

INTRODUCTION

Bacteriocins are bactericidal agents produced by many species of bacteria. Recently, electron microscopic studies have revealed that the structure of some bacteriocins resembles a phage-like object or phage component such as the tail assembly. (Endo et al. 1965; Meningmann, 1965; Sandoval, Reilly & Tandler, 1965; Ishii, Nishi & Egami, 1965; Bradley & Dewar, 1966; Higerd, Baechler & Berk, 1967; Bradley, 1967; Lang, McDonald & Gardner, 1968; Coetzee et al. 1968).

Ishii et al. (1965) showed that a trypsin-resistant pyocin, a bacteriocin of Pseudomonas aeruginosa closely resembles T-even phage tails with a contractile sheath. Takeya et al. (1966) isolated a small pyocin with a different morphology and designated it pyocin 28. The present paper describes the properties of pyocin 28.

METHODS

Bacterial strains. The pyocinogenic Pseudomonas aeruginosa strain P28 was used for pyocin production and strain P29 as an indicator. Both strains were kindly supplied by Dr J. Y. Homma of Institute for Medical Science, Tokyo University.

Production of pyocin 28. Log.-phase bacteria of the strain P28 were heavily inoculated in nutrient broth containing mitomycin C (1 µg./ml.) and incubated for 3 hr with constant shaking. The concentration of mitomycin added was determined by preliminary experiments. The time course of pyocin production under these conditions was described by Takeya et al. (1966).

Assay of pyocin 28 activity. Two ml. of 0.7% agar containing 10⁷ indicator bacteria were layered on to base agar medium. A loopful of each serially diluted pyocin sample was spotted on the agar plate and incubated overnight at 37°. The pyocin unit was expressed as the highest dilution of pyocin inhibiting the growth of the indicator strain.
Preparation of pyocin. The pyocin was purified at temperatures < 10°C. The pyocin lysate was centrifuged at 10,000 rev./min. for 30 min. in order to remove bacteria and bacterial debris. The supernatant fluid was salted out by saturation with ammonium sulphate. The pyocin was collected as a floating fluffy surface layer, as seen in the purification of rod-shaped phage of Pseudomonas aeruginosa (Takeya & Amako, 1966). The surface layer was suspended in 0.01 M-tris buffer (pH 7.4) + 0.1 M-NaCl and dialysed against buffered saline of the same composition. After dialysis, the contents were centrifuged at 10,000 rev./min. for 30 min. and the supernatant fluid was again centrifuged at 55,000 rev./min. for 90 min. The pellet obtained was resuspended in a buffered saline solution and was purified by 3 cycles of differential centrifugation. The final sample was designated as partially purified pyocin.

Some batches were further purified by column chromatography on DEAE-cellulose or sucrose density gradient centrifugation, in order to identify the pyocin activity with rod-shaped particles. Three and a half ml. of partially purified pyocin containing approximately 20 mg. of total protein were loaded on the DEAE-cellulose (Serva) packed in a column 1.0 x 16.0 cm. After washing with 0.01 M-tris buffer (pH 7.5), elution was carried out at first by 0.1 M-NaCl + 0.01 M-tris buffer (60 ml.), and then by linear gradients between 0.1 and 0.4 M-NaCl + tris buffer (200 ml.). Each eluted fraction was assayed for absorbency at 280 nm. pyocin activity and presence of pyocin rods by electron microscopy. Sometimes, fractions with greatest pyocin activity were collected and repurified by chromatography. One tenth ml. of partially purified pyocin was layered on to 4.5 ml., 10 to 30% sucrose + tris + NaCl buffer gradients and centrifuged for 120 min. at 5 to 10°C, at 35,000 rev./min., in the SW 39 rotor of a Spinco Model L ultracentrifuge. Fourteen fractions (each approximately 0.3 ml.) were collected by needle puncture at the bottom of the tube. Each fraction was assayed for pyocin activity and examined for the presence of pyocin rods by electron microscopy.

Enzyme treatment. Purified pyocin was suspended in tris-buffered saline and pyocin activity was measured before and after treatment with various enzymes at 37°C for 1 hr. Trypsin (Difco Lab. Inc. Detroit, U.S.A., 1:250) (2 mg./ml.) Nagarse (Teikoku Chemical Industry Co., Osaka, Japan), a bacterial proteolytic enzyme, (0.2 mg./ml.), DNase (Nutritional Biochemicals Co., Ohio, U.S.A.) (0.2 mg./ml.) and RNase (Nutritional Biochemicals Co., Ohio, U.S.A.) (0.2 mg./ml.) were used.

Heat and ultrasonic treatment. Purified pyocin was suspended in tris-buffered saline, and pyocin activity was assayed after heating for 10 min. at temperatures between 40° and 80°. Pyocin activity was also assayed after ultrasonic treatment (10 kcal./sec., 200 w) for different times, during which the sample was kept cool < 35°C with running tap water. Treatment for 30 min. was given as three successive 10 min. exposures with intervals of 10 min. each to prevent temperature elevation.

pH stability. A thick pyocin suspension was diluted with various buffers to obtain suspensions of different pH values and pyocin activity was assayed after incubation for 30 min. at 37°C. A 0.01 M-citrate buffer was used for pH 2.6 to 7.0, 0.01 M-tris + HCl buffer for pH 8.0 to 9.0 and 0.05 M-Na2CO3 solution for pH 11.0.

Electron microscopy. A purified pyocin suspension was mixed with equal volume of 2% neutral potassium phosphotungstate solution and applied to carbon film on sheet meshes. Bacterial suspension treated with the pyocin was also prepared in the same way. Specimens were examined in a JEM 7 electron microscope.
(a) and (b) Partially purified pyocin 28. Arrows indicate closed sharp ends of the pyocin rods.

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(a) An indicator cell of *Pseudomonas aeruginosa* (P29 strain) after treatment with pyocin 28. Numerous pyocin rods attach to the cell.

(b) Attachment of pyocin rods to the bacterial surface of *Pseudomonas aeruginosa* (P29 strain).

(c) A cell of pyocin-sensitive *Pseudomonas aeruginosa* (K2 strain) after treatment with pyocin 28. Numerous pyocin rods attach to the cell surface.

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**Partial purification of pyocin 28**

Usually 10,000 to 100,000 units of activity were attained after partial purification by chemical methods and differential centrifugation. Partially purified samples sometimes contained bacterial flagella, but the flagella could be separated by DEAE-cellulose column chromatography. After chromatography, the presence of numerous pyocin rods was specific to the fractions with high pyocin activity. The close correlation of the rods with the active principle of pyocin 28 was indicated also by the results of density gradient centrifugation. Numerous pyocin rods and rings were found specifically in the fractions with high pyocin activity (nos. 8, 9 and 10), while pyocin rods were scarce in fractions which showed little or no pyocin activity (nos 1 to 5 and nos 11 to 14). An electron micrograph of the fraction with highest pyocin activity (no. 9) obtained by sucrose density gradient centrifugation showed many pyocin rods about 1000 Å long and ring-shaped segments (Pl. 2a).

**Stability of pyocin 28**

The pyocin activity was resistant to treatment with trypsin, Nagarse, DNase and RNase. By ultrasonic treatment the pyocin activity was scarcely affected for the first 10 min. but was slightly reduced after 30 min. The pyocin was stable at 50° but was completely inactivated by heating at temperatures greater than 60° for 10 min. It was stable at 37° between pH 5.0 and 8.0 but unstable at pH values below and above this range.

**Electron microscopy**

The width of the pyocin was uniform, measuring about 90 Å (Pl. 1a, b). The length, however, was not uniform varying from 200 to 4000 Å, but rods measuring 1000 to 12000 Å were most frequent (Fig. 1). Regular cross-striations, at centre to centre intervals of 35 Å, were clearly seen on the rods. In some of the rods both ends were square but many rods had one sharp end. A fine fibre, measuring about 200 Å, sometimes extended from the tip of the sharp end (Pl. 2b). A dark central line was usually observed in the rods but the line was hardly visible in the part near the sharp end; perhaps because potassium phosphotungstate which had entered the central hole through the open square end hardly reached the sharp closed end. Scattered small rings, 90 to 100 Å in diameter, were frequently observed. They probably represent small disrupted segments.

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**PLATE 2**

(a) Pyocin 28 purified by sucrose density gradient centrifugation. No. 9 fraction with highest pyocin activity obtained after 10 to 30 % sucrose density gradient centrifugation for 120 min. at 35,000 rev./min. in the SW 39 rotor of Spinco Model L ultracentrifuge.

(b) Partially purified pyocin 28. Arrows indicate a fine fibre extending from the tip of the sharp end of the rod.

(c) Pyocin 28 treated with alkali (pH 11.0) for 24 hr. Many pyocin rods swollen and disrupted.

(d) A core of a pyocin which closely resembles headless phage tail with contractile sheath.

(e) A rod of pyocin 28 with a clearly visible central dark line.

(f) Rods of pyocin 28 with cross-striations.

(g) A tail of mycobacteriophage B-1.

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of the rods standing upright. After 24 hr incubation at pH 11.0, many rods were disrupted and the number of small rings markedly increased. Moreover, rods and rings with somewhat swollen unloosed protein subunit structures were often observed (Pl. 2c).

Purified pyocin was mixed with cell suspensions of various strains of *Pseudomonas aeruginosa* and the mixtures were examined by the electron microscope after incubation at 37° for 30 min. No pyocin rod was observed on the cell surface of *Pseudomonas* strains insensitive to pyocin 28, while numerous pyocin rods could be seen to be attached to the cell surface of the pyocin-sensitive strains (Pl. 3a, b, c). The pyocin rods were attached to the cell perpendicularly, probably by their sharp ends since free ends were always observed as square.

Fig. 1. Distribution of length of pyocin rods. Measurement was made on 240 pyocin rods.

**DISCUSSION**

Bradley (1967) tentatively classified bacteriocins into two groups. One was trypsin-resistant, thermolabile and consisted of particles with definite structures which may represent defective phages including headless tails; the other contained trypsin-sensitive and thermostable smaller molecules which could hardly be visualized by electron microscopy. Since pyocin 28 was found to be trypsin-resistant, thermolabile and to have a definite rod structure, it will be classified with Bradley’s first group of bacteriocins. However, pyocin 28 has a unique structure different from those of the first group of bacteriocins previously reported. If pyocin 28 represents a defective phage or a portion of a phage, there are three possible explanations for the origin of the pyocin from the standpoint of its morphology and dimensions. The fibrous shape and the width of the pyocin resemble those of fibrous *Pseudomonas* phage Pf isolated by Takeya & Amako (1966). Thus, at first the pyocin was suspected to be a defective form of the Pf type of fibrous phage. However, there was no serological relationship between the pyocin and Pf phage, and structures resembling the pyocin could not be produced.
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from Pf phage rods in spite of various chemical and physical treatments (Takeya, unpublished data). On the other hand, it was noticed that the structure of the pyocin with a central dark line resembled that of the core of the pyocin of T-even phage-tail type (Pl. 2d, e). However, the fact that the pyocin 28 was often slightly curved, while the core was always straight, suggests that these two entities were different.

The third possibility was that the pyocin was a sheathless tail of phage. The fine structures of the pyocin closely resembled those of the long sheathless tail of phages of group B (Bradley, 1967). Although the sheathless tail in Pl. 2g presented for comparison belongs to a mycobacteriophage (Takeya & Amako, 1964), many Pseudomonas phages with sheathless tail have been isolated, including B3 reported by Slayter, Holloway & Hall (1964). The length of the pyocin is not uniform but rods of 1000 to 1200 Å are most frequent. These rods may represent the normal tail length and longer rods may be polytails. The occasional observation of a fine fibre resembling a tail fibre of coliphages (Bradley, 1964; Eiserling & Boy de la Tour, 1965) at the sharp end of the pyocin supports this view.

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REFERENCES


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