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ISOLATION AND CHARACTERIZATION OF PYOCINS FROM SEVERAL STRAINS OF *PSEUDOMONAS AERUGINOSA*

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Two types of pyocins were found in *Pseudomonas aeruginosa* and named pyocin type S and type R. Production of both types of pyocin was inducible by ultraviolet ray or by mitomycin. An S-type pyocin was prepared from strain P28 and its nature was investigated. The pyocin activity was labile to heat or proteinase treatment. Its molecular weight was estimated to be around 1×10^5. The same type of pyocin was found in M11, a fertile strain originally isolated by Holloway.

Pyocin type R, which has a structure very similar to some phage tails, was found among three strains of Japanese and Australian origin and named pyocin R2, R3, and R4. These pyocins distinguished by the difference in their action spectrum but were otherwise indiscernible. They behaved in the same manner during the purification process including DEAE-cellulose chromatography. Moreover an immunological cross reaction was observed among these R-type pyocins. Anti-pyocin R or R2 serum also neutralized other R-type pyocins. Electron microscopy revealed a similar dimension for each pyocin particle; 150 Å×1,300 Å.

Bacteriocins are bactericidal substances of protein nature produced by certain bacteria and are active against some other strains of the same or closely related species. Some 20 kinds of bacteriocin have so far been reported (*1*, *2*). However, detailed chemical studies on bacteriocins are rather rare.

Bacteriocinogeny in *Pseudomonas* has been observed by many workers (*3–5*). One of us has purified a pyocin, pyocin R, which was revealed to have a structure similar to some phage tails (*6*). The chemical, physical, or biological characterization of the pyocin has been made and its resemblance to phages became more conspicuous (*7–9*). Similar structures were found by BERK *et al.* (*10*) and by BRADLEY (*11*). We started to search for pyocins among several other strains of *Pseudomonas aeruginosa* for the purpose of

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comparative study of pyocin. So far we have found two types of pyocin, type R and type S. The former is a very large particle which is sedimentable by ultracentrifugation and has a structure like pyocin R. The latter is not sedimentable, not visible in the usual electron microscopy, and sensitive to proteinases.

A fertility factor was found in the *Pseudomonas* by HOLLOWAY (*12*), and the genetic analysis has been carried out with this mating system (*13*, *14*). We found both types of pyocin in the fertile strains, one of which was very similar to pyocin R.

This paper describes the preparation and characterization of one S-type pyocin and three R-type pyocins, R2, R3, and R4, which look very similar but are distinct from each other.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are listed in Table 1. Those prefixed with M are derived from Holloway's strains which were given to us through Dr. Tsutomu Watanabe of Keio University. Those prefixed with P are derived from strains originally isolated by Dr. J. Y. Homma of the Institute of Medical Science, University of Tokyo. Pyocins were found in strains M11, M2008, and P28, and characterized in this study and named as listed.

Culture conditions. Either nutrient broth or G medium was employed for the bacterial cultivation and preparation of pyocins. Nutrient broth was composed of 10 g of polypeptone (Daigo Eiyo Co., Osaka), 10 g of meat extract (Kyokuto Co., Tokyo), and 1 g of NaCl in 1 liter. pH was adjusted to 7.0 with 1 N NaOH. G medium contained 20 g of sodium glutamate, 5 g of glucose, 0.1 g of $MgSO_4 \cdot 7H_2O$, 5.63 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of KH_2PO_4, and 0.5 g of yeast extract (Daigo Eiyo Co.) in 1 liter. When amino acid auxotrophic mutants were cultured, the required amino acids were added in a concentration of 50 μg each per ml. Kyokuto nutrient agar was used for solid plates.

Detection of pyocinogeny on agar plates was performed as follows. Test strains were spotted with needles on nutrient agar plates and incubated for several hours until small colonies appeared. Then they were irradiated with an UV lamp to induce the pyocin production (15-W germicidal lamp, 70 cm for 1 min). After several hr of incubation, surviving colonies were killed with chloroform vapor and removed by impressing on sterile velvet. Resulting plates were layered with 2 ml of soft agar solutions (0.5%) containing about 5×10^7 cells of an appropriate indicator strain. After overnight incubation, clear inhibitory zones were observed around pyocinogenic colonies.

The assay method of pyocin activity in solution (pyocin unit) was the same as described previously (*5*).

Preparation of pyocin lysate. Strains M11, M2008, and P28 were found

Table 1. Strains used.

a) Pyocinogenic strains

Strain	Pyocinogeny		Auxotrophy	Origin
	R-type	S-type		
M11	R2+	+	*trp*	Holloway's strain 1, *str*^{r}, FP$^-$
M2008	R3+	−	*ile* and *val*	Holloway's strain 2, *str*^{r}, FP$^+$
P15	R+	−	prototroph	Homma
P28	R4+	+	prototroph	Homma

b) Indicator strains

Strain	Character	Origin
P4, P11, P14	prototroph	Homma
P11R^r	prototroph	P11, spontaneous pyocin R resistant
P15–16	trp, str^r, pyR^{-a}	P15, NG^b treatment
P15–16R^r	trp, str^r, pyR$^-$	P1516, spontaneous pyocin R resistant
P15–16R2^r	trp, str^r, pyR$^-$	P1516, spontaneous pyocin R2 resistant
P15–16S^r	trp, str^r, pyR$^-$	P1516, spontaneous pyocin S resistant
P15–16PS3^r	trp, str^r, pyR$^-$	P1516, spontaneous phage PS3 resistant
M78	trp, arg, str^r, pyR2^{-a}	M11, NG^b treatment

^{a} pyR or pyR2: Productivity of pyocin R or R2.
^{b} NG: N-Methyl-N′-nitro-N-nitrosoguanidine.

to produce pyocins after induction by the addition of Mitomycin C as well as by the ultraviolet irradiation in liquid culture. The pyocin-rich lysate was prepared as follows: Overnight culture was diluted 50–100 times with a fresh medium and the culture was carried out at 37° for 2–3 hr under constant shaking. At the logarithmic phase of growth, 2 μg/ml of Mitomycin C was added. Turbidity of the culture increased for about 90 min until the cell lysis began. The clear lysate was obtained about 3 hr after the addition of Mitomycin C.

Purification of pyocins. The procedure for the purification of pyocin R reported by KAGEYAMA (*6*) was adopted with a slight modification to purify other pyocins. Mytomycin-induced lysate was treated with DNase (1 μg/ml) for 1 hr at 37° and the bacterial debris was removed by centrifugation. All R-type pyocins were found to be adsorbed by the DEAE-cellulose chromatography, while pyocin S activity was not retained by the column.

Preparation of antiserum. Purified pyocin R and R2 were used as antigens and the antisera were prepared as described previously (*7*).

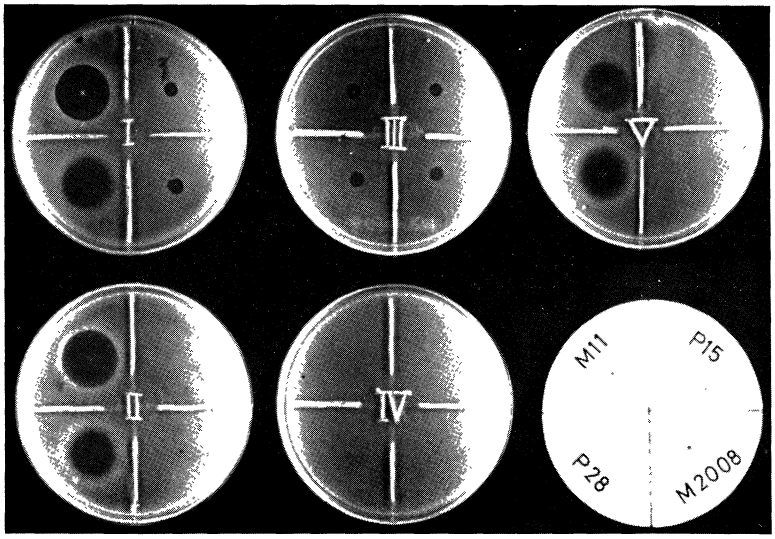

Fig. 1. Detection of pyocinogeny of various strains.

Pyocinogenic strains, M11 (pyocin R2 and type S), P15 (pyocin R), P28 (pyocin R4 and S), and M2008 (pyocin R3) were spotted on agar plates (I–V) as indicated (bottom right).

Indicator strains were: I, P15-16; II, P15-16 plus 0.1 ml of antipyocin R serum; III, P15-16S^r; IV, P15-16S^r plus 0.1 ml of anti-pyocin R serum; V, P15-16R^r.

RESULTS

Detection of pyocin S activity

As shown in Fig. 1, some strains of *Pseudomonas aeruginosa* (M11, P28) were found to produce pyocins which showed much broader inhibitory zones than that of pyocin R on agar plates (Fig. 1, Plate I, II, V). The active principle responsible for the broader inhibition will be referred to as S-type pyocin. A mutant resistant to pyocin S (P15-16S^r) was found to be sensitive to another kind of pyocin which shows narrower inhibitory zones and is produced by all four strains (Plate III). The active principle responsible for the narrower inhibition will be referred to as R-type pyocin. A mutant was obtained (P15-16R^r) which was resistant to all the R-type pyocins produced by strains P15, P28, M11, and M2008. This strain (P15-16R^r) was found to be sensitive to pyocin S (Plate V). Thus, it is clear that S- and R-type pyocins differ in the receptor specificity as well as in their diffusion pattern on agar plates. P15 and M2008 were found to produce R-type pyocin, and P28 and M11 produced both types of pyocin, as far as this assay system was concerned.

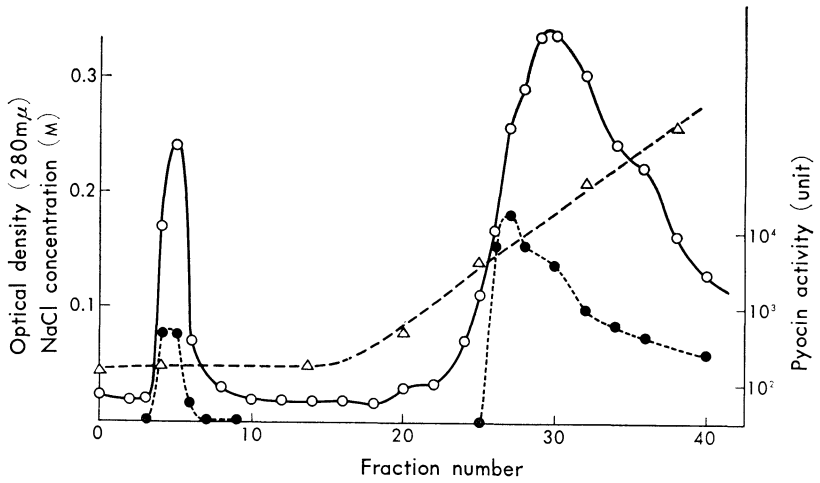

Fig. 2. Chromatographic profile of a pyocin preparation of P28 on DEAE-cellulose.

A pyocin sample (pyocin S=1.1×10^4 units, pyocin R=1.1×10^6 units) was charged on a DEAE-cellulose column (2 cm×37 cm) and was eluted with a gradient concentration of NaCl containing 0.01 M Tris buffer (pH 7.5). The flow rate was 40 ml/hr, 1 fraction was 12 ml.

○—○ Optical density at 280 mμ.

△--△ Chloride concentration in the eluate.

●--● Pyocin activity measured with P11R^r for pyocin S and P4 for pyocin R4.

The recovery of pyocin S and pyocin R4 activity in this chromatography was 85% and 52%, respectively.

Partial purification of pyocin S

Strain P28 was used for the characterization of two types of pyocin, which were named pyocin S and pyocin R4. The Mitomycin-induced lysate of P28 generally showed about 3×10^3 units of both activities. Ammonium sulfate was added to a DNase-treated lysate (80% saturation). The precipitate was collected and dissolved in and dialysed against 0.01 M Tris buffer containing 0.05 M NaCl (pH 7.5). The protein solution was charged on a DEAE-cellulose column prewashed with the same buffered saline. Pyocin S activity passed through the column while pyocin R4 activity was retained and eluted by the gradient increase of NaCl concentration (Fig. 2). Thus two activities were easily separated. The recovery of pyocin S activity at this stage was about 20–30% of the lysate. An attempt for further purification of pyocin S was difficult. Chromatography with CM-cellulose resulted in a great loss of the activity.

Properties of pyocin S activity

The effluent fraction of the DEAE-cellulose chromatography was used to

study the properties of pyocin S activity. The activity was completely destroyed by heat treatment (10 min incubation at 60°) or by proteinase digestion (incubated with 100 μg/ml of either trypsin, chymotrypsin, or Nagarse at pH 7.5, 30° for 1 hr). The pH stability was studied. Incubation at 37° for 30 min caused no loss of activity in a pH range from 7 to 10, but considerable inactivation occurred outside of this pH range.

The molecular weight of pyocin S was estimated by gel filtration through Sephadex G200 using horse hemoglobin as a reference. As expected from the behavior on agar plates (*i.e.*, broader diffusion), the molecular weight of pyocin S was much lower than that of pyocin R. It was estimated to be around 10^5.

Pyocin S activity was not neutralized by the anti-pyocin R serum, (Fig. 1, Plate II) and was not sedimented by ultracentrifugation ($60{,}000 \times g$, 45 min). S-type pyocin was also recovered by the same procedure from the Mitomycin-lysate of strain M11.

R-type pyocins

The occurrence of R type pyocins in strains of *Ps. aeruginosa*, M11, M2008, and P28 was suggested from the detection plate (Fig. 1). Electron microscopic observation of these lysates revealed that each strain produced a similar structure like pyocin R. Moreover, it is evident that these pyocins have some immunological relationship to each other, as they were all neutralized by the anti-pyocin R serum (Fig. 1, Plate IV). However they could be distinguished from each other by the difference in their action spectrum. R type pyocins of these strains (M11, M2008, and P28) will be referred to as pyocin R2, R3, and R4 respectively.

Action spectrum of R type pyocins

The killing activity of these pyocins against several *Pseudomonas* strains is summarized in Table 2. Thus, these pyocins can be distinguished from each other by using appropriate indicator strains. It is evident that a pyocinogenic strain is not killed by its own pyocin.

Isolation and purification of pyocins R2, R3, and R4

All R-type pyocins showed a very similar behavior in the purification procedure. About 1×10^4 units of R-type pyocin activity was found in both M11 and M2008 lysate. A chromatographic profile of M11 pyocins is shown in Fig. 3. Pyocin R2 activity was recovered at the chloride concentration between 0.15 and 0.16 M, while S-type pyocin activity passed through the column. The profile was essentially the same as that of P28 pyocins (Fig. 2) and of M2008. Thus pyocins R2, R3, or R4 are all eluted from the DEAE-cellulose column at the chloride concentration of 0.15–0.16 M. The activity was recovered entirely from the peak fractions by ultracentrifugation (60,000

Table 2. Action spectra of pyocins.

Pyocin (produced by) / Strains	R (P15)	R2 (M11)	R3 (M2008)	R4 (P28)	S (P28)
P11, P14, P15–16	s	s	s	s	s
P15–16S^r	s	s	s	s	r
P15	r	s	s	s	s
P4	r	s	s	s	r
M2008	s	s	r	s	s
M78	s	s	r	s	r
M15–16 PS3^r	s	s	r	r	s
P15–16R2^r	s	r	r	r	s
P11R^r, P15–16R^r	r	r	r	r	s
M11, P28	r	r	r	r	r

s: sensitive, r: resistant

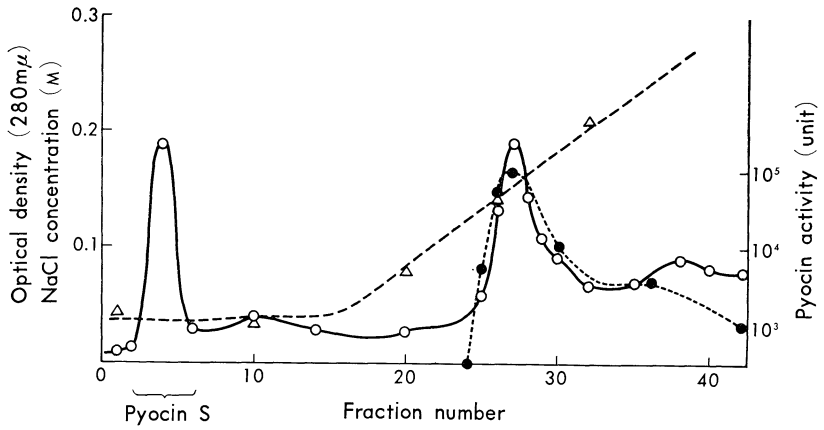

Fig. 3. Chromatographic profile of a pyocin preparation of M11 on DEAE-cellulose.

Pyocin R2 sample (4×10^6 units) was charged on a column (1.4 cm × 22 cm). The elution was made by a linear gradient of NaCl. The flow rate was 40 ml/hr, and fractions were collected every 10 ml. P15–16S^r was used as an indicator.

○—○ Optical density at 280 mμ.

△--△ Chloride concentration in the eluate

●--● Pyocin activity.

The recovery of pyocin R2 activity in this chromatography was 70%. Pyocin S activity was not assayed quantitatively, but actually appeared in the non-adsorbed fraction as indicated.

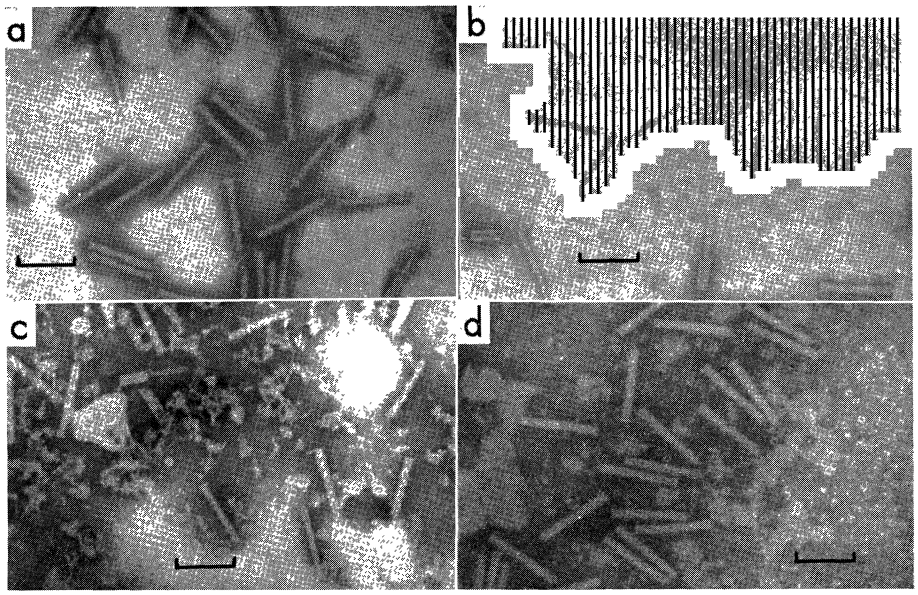

Fig. 4. Electron micrograph of R-type pyocins.

Preparations of pyocin were negatively stained with 2% neutral sodium phosphotungstate.

a) Pyocin R, b) Pyocin R2, c) Pyocin R3, d) Pyocin R4.

Pyocin R was a purified preparation. R2 and R3 were prepared by repeating low- and high-speed centrifugations (The chromatographic process was omitted). R4 was a sample prepared by the ammonium sulfate precipitation. Scales in the pictures indicate 1000 Å.

$\times g$, 45 min). Generally 20–50% of the activity in lysates was recovered in an essentially pure form.

Electron microscopy

Electron micrographs of four R-type pyocins are shown in Fig. 4. These pictures clearly indicate their resemblance to some phage tails. They are about 15 mμ in width and 120–130 mμ in length. Several structural components, such as core, sheath, base plate, etc., are observable in every sample. Incomplete particles are often seen in less purified preparations.

Immunological relationship of R-type pyocins

These four pyocins resembled not only morphologically but also immunologically. When 0.1 ml of anti-R serum was added to the soft agar of pyocin assay, no inhibitory zone was observed by pyocin R2, R3, or R4 as well as R, while pyocin S activity was not affected. (Fig. 1, Plates II, IV). The same situation was found with anti-R2 serum. A quantitative assay of

the immunological relationship of these pyocins will be given in a forthcoming paper.

DISCUSSION

Two types of pyocins were found among several *Pseudomonas* strains. One is a large particle which is sedimentable by ultracentrifugation and is resolved in electron microscopy as a phage tail-like structure. We will refer to this kind of pyocin as type R. Another kind of pyocin, referred to as S-type, is a smaller particle than R-type ones but apparently of protein nature.

S-type pyocin was found in strains M11 and P28. The molecular weight of pyocin S from P28 was roughly estimated to be 10^5 by the gel filtration method with Sephadex. Although purification of pyocin S is not yet established, it is most probably a protein, judging from its size and lability to heat or proteinase treatment. Production of pyocin S can be induced with Mitomycin C. Without induction, the activity is generally 10–20 units in the culture medium of either strain. After induction, S activity generally reaches 10^3 units in the lysate.

So far there seems no relation between S- and R-type pyocins. Pyocin S activity was not neutralized by anti-pyocin R serum. Receptors may also be different. Derivatives of strain P15-16 resistant to R-type pyocins are still sensitive to pyocin S (Fig. 1, Plate V) and *vice versa*. It is not clear by this time whether S-type pyocins of P28 and M11 are the same or different entities.

S type pyocin may belong to the same category as colicin E (*15*) or megacin (*16*). More detailed characterization of this type of pyocin is under way in our laboratory. A similar type of pyocin of low molecular weight, proteinase sensitive, was found by Homma *et al.* in the cell extract of *Ps. aeruginosa* strain Pl-III (*17*).

Four kinds of R-type pyocin were found which were very similar in structure and in their behavior in purification process. The occurrence of immunological cross-reaction among these pyocins suggests that there is some common or closely related component(s) in their structure. These pyocins are discernible by the difference in their action spectrum, the specificity of the killing action. This difference may correspond to that in the receptor structure, and this point will be examined in a forthcoming paper.

Pyocin R and R4 are produced by the strains originally isolated by J.Y. Homma in Tokyo, and strains which produce pyocin R2 or R3 were isolated in Australia. It is of some interest that these independent isolates elaborate pyocins so closely related. Similar structures were discovered by some authors in *Pseudomonas* after Mitomycin treatment (*10*, *11*). According to Bradley, the strain which was first described by Jacob to be pyocinogenic (*3*) also produced a similar structure after ultraviolet irradiation (*11*). Thus, R-

type particles seem to be widely distributed among the *Pseudomonas*. The same kind of bacteriocin has recently been discovered not only in *Pseudomonas* but also in other genera (*18—21*). This kind of substance may be best interpreted as defective phages. These killing particles could have originated from bacteriophages. Recently, HOMMA *et al.* (*22*, *23*) and we have discovered several bacteriophages which have some immunological relation to R-type pyocins. Comparison of physical, chemical, and biological properties of these entities will be interesting and informative in relation to the problem of the origin and evolution of pyocins.

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