In Vitro and In Vivo Characterization of Pyocin

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Pyocin, a bacteriocin obtained from lysates of ultraviolet-induced cultures of *Pseudomonas aeruginosa* was characterized in vitro and in vivo after 1,000-fold purification by chemical, column, and differential centrifugation procedures. Electron micrographs of negatively stained pyocin preparations contained rod-shaped particles which resembled the contractile tail protein of the T-even phages of *Escherichia coli*. Although two separate and distinct pyocin fractions were eluted from diethylaminoethyl cellulose (pH 7.5) during the purification procedure, the particles appeared identical. In addition, the two fractions exhibited a close correlation between their titers and the particle numbers as observed in the electron microscope. The particles were approximately 20 by 90 m μ with a core diameter of 5 m μ and a sheath length of 50 m μ . Neither intact phage nor ghosts were seen in any of the preparations, although ringlets of two different diameters, which appeared to correspond to the diameters of the sheath and inner core, were observed. Other studies indicated that, although crude preparations were stable to freezing and thawing, purified preparations lost all of their activity under similar treatment. However, the addition of 50% glycerol to purified preparations completely protected activity. Conversely, aged normal human or rabbit sera enhanced the antibacterial activity of pyocin approximately fourfold, although serum albumin and hemoglobin had no effect. In vivo studies indicated that purified pyocin was not lethal for mice when injected intraperitoneally in concentrations of 28,000 to 1,400,000 units (5.6 to 276 μ g of protein), nor was 7,200 to 36,000 units dermonecrotic for rabbits.

Bacteriocins are antibiotics produced by many bacteria that kill sensitive organisms of the same or closely related species. Pyocin, a bacteriocin of *Pseudomonas aeruginosa*, was first described by Jacob (17), who showed its synthesis was inducible by irradiation with ultraviolet (UV) light; later studies by Hamon (12) indicated the existence of protease-sensitive and -resistant varieties. More recently, Kageyama and Egami (19) have purified the protease-stable pyocin 100-fold. Electron microscopic studies indicated a structure of rod-like particles of uniform size (18). More refined electron microscopic studies by Ishii et al. (16) indicated that the main structure of pyocin closely resembles T-even phage tails which consists of a contractile sheath and inner core. At the present time, studies on pyocin are of interest for a variety of reasons, one of which is from a host-parasite point of view with regard to the infectious processes in man, since Homma and Suzuki (13, 14) have shown that pyocin and the protein moiety of *P. aeruginosa* endotoxin are chemically and immunologically related, if not identical. Both are capable of

eliciting a pyrogenic and Shwartzman reaction in rabbits in equal potencies. Similar studies (1, 9, 10) with colicine K from *Escherichia coli* K235 indicate that it is serologically and chemically similar to the O antigen in this bacterial strain; however, the antibiotic activity resides in the protein moiety of the complex. A second area of interest is the possible relationship of bacteriophage with bacteriocins since they appear to have certain properties in common (8, 23). Consequently, the aim of this study is the in vitro and in vivo characterization of a bacteriocin from *P. aeruginosa* in order that its relationship to both phage and host-parasite studies be clarified.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* strain W₉ served as a sensitive indicator strain for the titration of pyocin produced by strain C₉.

Medium. The composition of the growth and titration medium consisted of 2% tryptone, 1% glucose, 0.5% sodium chloride, and 1.5% agar.

Pyocin production. An 18-hr culture of C₉ was suspended in sterile saline (0.85%) and was diluted so as to have an absorbancy of 0.50 at 660 m μ . Of this sus-

pension, 10 ml was added to 1 liter of 2% tryptone broth, and together they were incubated at 37 C for 3 hr under constant agitation. Samples of 50 ml of the broth culture were then exposed to UV irradiation for 40 sec in a Pyrex baking dish (205 by 340 mm) under constant agitation and at a distance of 30 cm from the light source (Westinghouse Sterilamp 782L-20). The treated broth was then reincubated in the dark for 3 hr at 37 C under constant shaking, at the end of which time the broth was centrifuged at $13,200 \times g$ for 30 min at 4 C in the Sorvall RC-2 Centrifuge. The supernatant fluid was designated as the crude broth lysate.

Pyocin assay. A modified assay system of Jacob (17) was used to titrate the pyocin activity of lysates. An 18-hr culture of the W₉ strain was suspended in sterile saline and was diluted so as to have an absorbancy of 0.10 on a Coleman Junior Spectrophotometer at 660 m μ . This standard suspension was diluted 1:5 with sterile saline. The addition of 0.25-ml samples of the diluted suspension was made to petri plates containing 20 ml of the 2% tryptone agar, and the organisms were spread over the agar surface by the use of a bent glass rod. Afterwards, the plates were incubated at 37 C for 3 hr, and one drop (0.05 ml) of each serially diluted pyocin sample was placed on the agar surface. The plates were reincubated at 37 C until the sensitive lawn first became visible, at which time the plates were read. The unit of pyocin activity was expressed as 20 times the reciprocal of the highest dilution which gave a completely clear zone of inhibition (0.05 ml of each dilution spotted on the agar).

Protein. Determination of protein in pyocin samples was based on the Zak and Cohen (31) modification of the Lowry method. Bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was used in the construction of the standard curve.

Pyocin purification. Purification of pyocin in lysates was performed according to a modification of the method of Kageyama and Egami (19). Of the cell-free lysate, 1 liter was treated with 60 ml of 1 M MnCl₂ and was adjusted to pH 7.5 under vigorous agitation prior to centrifugation at $13,200 \times g$ for 30 min. To the supernatant fluid, 500 g of ammonium sulfate was added, and the mixture was refrigerated for at least 2 hr. The precipitate was then centrifuged at $30,000 \times g$ for 15 min at 4 C and was dissolved in 50 ml of 0.02 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (pH 7.5), containing 0.01 M magnesium chloride and 0.01 M magnesium sulfate. After dialysis for at least 24 hr against 2 liters of the same buffer, the pyocin solution was cleared by centrifugation at $8,000 \times g$ for 15 min at 4 C. The solution containing pyocin was then centrifuged in the Beckman Model L Preparative Ultracentrifuge for 60 min at $100,000 \times g$ with the type 50 rotor. The jelly-like pellet obtained was gently dissolved in the same Tris chloride buffer and was chromatographed on a diethylaminoethyl (DEAE)-cellulose (Calbiochem, Los Angeles, Calif.) column which had previously been washed with 2 liters of the Tris chloride buffer (pH 7.5). After the pyocin sample was applied to the column (25 by 150 mm) for 1 hr, 200 ml of the Tris chloride buffer was passed to eliminate any substance not readily adhering to the column material. Subsequently, 800 ml of a

sodium chloride gradient (0 to 1.0 M sodium chloride in the 0.02 M Tris chloride buffer) was applied in a linear manner. The resulting chromatographic fraction containing pyocin activity was then dialyzed against the same buffer to remove the sodium chloride. The dialysate was designated as the purified pyocin solution. Concentrated solutions necessary for animal inoculations and electron microscopy studies were obtained by ultracentrifugation as previously described.

In vivo studies. Determination of dermonecrotic activity of purified pyocin preparations was performed in triplicate by injecting 0.2 ml of various dilutions (7,200 to 36,000 units) subcutaneously into rabbits (New Zealand strain). Control rabbits received an equal amount of the suspending Tris chloride buffer. The injected areas were observed daily for dermonecrosis over a 3-day period. The possible lethal effect of purified pyocin on mice was determined in quadruplicate by injecting the material intraperitoneally (28,500 to 1,440,000 units). Injected mice were observed at several time intervals over a 72-hr period. All in vivo studies employed pyocin which had been sterilized by treatment with UV for 2 min.

Electron microscopy. Negative-contrast methods (3, 4, 11, 25) which have been described for the purpose of studying the ultrastructure of virions were employed in the study of pyocin. Uranyl acetate (0.5%) or silico-tungstate (1.5%) was used as the negative stain upon which the collodion pseudoreplicas were floated from agar blocks. These pseudoreplicas were placed upon 400-mesh copper grids, and a thin film of carbon was evaporated over the surface to stabilize the membranes. Grids were examined in a modified RCA EMU-3E electron microscope at an instrumental magnification of 32,000 diameters and at an operating voltage of 50 kv. The electron microscope was modified to include a specimen chamber "pump out," a heated platinum 20- μ objective aperture, and the standard high-voltage stabilization unit.

Effect of normal sera and serum components on activity. Supplements of 0.5 ml of various sera were added to 0.5-ml samples of a purified pyocin solution to effect a final concentration of 0.1, 1.0, 5.0, 10, and 25%. The normal sera tested included bovine, calf, chicken, horse, human, and rabbit sera. After 1 hr of incubation at 37 C, the mixtures were assayed for pyocin activity. Hemolyzed human whole blood and hemolyzed sheep blood were also tested in the same manner. In addition, purified human hemoglobin and human albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) were added to samples of pyocin in a final concentration of 2.0 and 20.0 μ g/ml. These mixtures were incubated at 37 C for 1 hr and were assayed for pyocin activity.

RESULTS

Initial studies were designed to determine whether an uninduced pyocinogenic strain of *P. aeruginosa* (C₃) spontaneously produces detectable pyocin titers during growth. As shown in Fig. 1, pyocin was first detectable in the growth medium at about 8 hr. From 8 to 24 hr, the titer

rose from approximately 40 to 1,200 units/ml, and its production generally corresponded to the growth response of the pyocinogenic strain. However, when a 3-hr broth culture was treated with 40 sec of UV irradiation or 1 μ g of mitomycin C per ml, then pyocin titers of 40,000 to 60,000 units/ml were obtained within 60 to 120 min after induction. Consequently, all subsequent studies were initiated with UV-induced cultures.

Prior to characterization of pyocin, broth lysates were purified by the modified method described in Materials and Methods. Pyocin was purified 1,030-fold with a recovery of 22.5% of total activity by use of chemical, differential centrifugation, and column procedures (Table 1).

FIG. 1. Spontaneous release of pyocin during growth of a noninduced culture of *Pseudomonas aeruginosa* (C₉).

The elution profile of pyocin from DEAE cellulose (pH 7.5) is presented in Fig. 2 and indicates the presence of two protein peaks which exhibit pyocin activity. The majority of activity was in the first peak after initiation of the sodium chloride gradient; it exhibited a 280 to 260 $m\mu$ ratio of 1.35, and the second gradient peak had a 280 to 260 $m\mu$ ratio of 0.7. Electron microscopic examination of negatively stained material sedimented from each peak at 100,000 $\times g$ for 60 min indicated the presence of rod-shaped particles which were identical in appearance. The pyocin titer of the second peak was approximately 1,000-fold less than that of the first gradient peak, and the relative pyocin particle numbers of both preparations also differed approximately 1,000-fold. Consequently, there was good correlation between pyocin activity and the visually observed particle numbers of the two preparations, suggesting that the observed particles were responsible for pyocin activity.

Electron micrographs of the structure of pyocin prior to column chromatography and after final purification can be seen in Fig. 3 and 4, respectively. The purity of the precolumn pyocin preparation was approximately 500-fold, and the appearance of these particles appeared to resemble the tail protein of T₄ coliphage in size and shape (30). In addition, they were morphologically identical to, but varied somewhat in size with, a pyocin preparation previously described (16). The majority of the particles in Fig. 3 measured approximately 20 by 90 $m\mu$ with a core diameter of 5 $m\mu$, and the length of the sheath was about 50 $m\mu$. Two strands of what is believed to be flagella from *P. aeruginosa* are also present in Fig. 3, in addition to a few sheaths which appear devoid of cores and seem to be paired together end-to-end. None of the electron micrographs of precolumn and postcolumn pyocin preparations exhibited the presence of

TABLE 1. Summary of purification data

Treatment	Vol (ml)		Pyocin activity		Protein (mg/ml)	Specific activity ^a	Recovery (%)	Purification (fold)
	Initial	Recovered	Units/ml	Total units				
Induced broth lysate	1,800	1,745	14,000	2.44×10^7	14.4	972	—	—
MnCl ₂	1,745	1,810	—	—	—	—	—	—
(NH ₄) ₂ SO ₄	1,810	105	120,000	1.26×10^7	12.5	9,600	51	9.8
Dialysis	105	141	80,000	1.13×10^7	3.7	21,620	46	22.0
Centrifugation at 100,000 $\times g$	141	15	700,000	1.05×10^7	1.4	500,000	43	514.0
Chromatography, centrifugation at 100,000 $\times g$	15	64	80,000	5.12×10^6	0.08	1,000,000	22.5	1,030.0

^a Expressed in units per milligram of protein.

intact phage, phage heads, or ghosts. In Fig. 4, the appearance of the particles in the final purified pyocin preparation resembles that of the precolumn pyocin particle in that two intact particles plus empty sheaths joined end-to-end are visible. Of all particles seen in the precolumn and post-column preparations, 90% consisted of contractile rod-shaped forms, and the remainder con-

sisted of partially degraded rods and empty sheaths. In addition to the rod-shaped forms, ringlets of two different diameters (5 and 20 $m\mu$) were frequently observed in most purified preparations and corresponded in size to the diameters of the cores and sheaths, respectively. Figure 5 shows the presence of four small ringlets.

In Fig. 6, some of the fine structure of purified pyocin can be observed. It is suggested that the particle consists of double hollow cylinders with cross striations. In addition, the unusual length of some of the particles seen appears to be due to two cores situated in close proximity to one another.

Because of the close immunological relationship between the protein moiety of *P. aeruginosa* endotoxin and pyocin (13, 14), both crude lysates and purified preparations of pyocin were examined in vivo. Mouse toxicity studies employing intraperitoneal injections of purified pyocin indicated no lethality over a 72-hr period when concentrations of 28,000 to 1,400,000 units (5.6 to 276 μg of protein) were administered. No lethality was noted with crude broth preparations consisting of equal protein concentra-

FIG. 2. Column chromatography of the partially purified pyocin preparation on diethylaminoethyl cellulose, pH 7.5.

FIG. 3. Electron micrograph of a partially purified pyocin preparation stained with silicotungstate. Two flagella may be seen traversing the field. The numerous contractile rod-like particles are believed to be responsible for pyocin activity. $\times 195,000$.

FIG. 4. Electron micrograph of a purified pyocin preparation stained with uranyl acetate. Two intact pyocin particles and two empty sheaths joined end-to-end are visible. $\times 340,000$.

tions. In addition, no dermonecrosis was observed with purified preparations in rabbits which had received 7,200 to 36,000 units (1.44 to 7.2 μg of protein) subcutaneously.

Purified pyocin was first characterized in vitro by thermal lability studies by use of a standard solution consisting of 4,000 units/ml for this and all subsequent studies. Pyocin was found to be stable at room temperature and at 4 C over a 15-day incubation period. However, when pyocin was frozen for any length of time, a complete loss of activity was noted. No morphological alterations were detected in frozen and thawed preparations when examined by electron microscopy. On the other hand, the activity of unpurified broth lysates was unaffected by freezing and thawing. Supplements of 50% glycerol completely protected purified preparations against freezing and thawing, whereas 10^{-3} M cysteine had no protective effect. Since pyocin was sensitive to freezing or thawing, an attempt was also made to determine whether activity would be lost after passage through the French pressure cell. Treatment of purified pyocin in this manner reduced the activity 10-fold. A shearing mechanism may

have been responsible for loss of activity in both procedures.

Another study concerning thermal lability indicated pyocin activity was completely retained after 10 min of incubation at 40, 50, and 60 C; however, no activity was retained at 70 C or higher. In addition, treatment of pyocin with UV for 2 min did not affect activity. Lipase (2 mg/ml), papain (2 mg/ml), trypsin (2 mg/ml), or *P. aeruginosa* purified protease (2 μg /ml; G. Johnson et al., Bacteriol. Proc., p. 40, 1966) also did not affect the activity of pyocin over a 3-hr incubation period.

To determine the effect of potential inhibitors on pyocin activity, supplements of various compounds were added to samples of purified pyocin and were incubated at 37 C for 1 hr at which time the activity was measured. No inhibition of activity was obtained with 10^{-3} M cyanide, 10^{-3} M ethylenediaminetetraacetate, 10^{-3} M cysteine, and 0.1 to 0.7 M sodium chloride. However, 8 M urea and 10^{-4} M *p*-chloromercuribenzoate (PCMB) abolished all activity, and 10^{-4} M mercuric chloride reduced the activity 95%. However, supplements of 1 M urea, 10^{-5} M PCMB, and

FIG. 5. Electron micrograph of a purified pyocin preparation stained with uranyl acetate. Four small ringlets may be observed (indicated by the pointer) as well as empty sheaths and intact particles. $\times 162,000$.

10^{-5} M mercuric chloride exerted little or no effect on activity.

Electron micrographs of urea and PCMB-treated pyocin preparations can be seen in Fig. 7 and 8. The studies with 8 M urea demonstrated a cleavage of sheaths into many large ringlet forms containing subunit structures which probably represent the smaller ringlet form (core). Concomitantly, with the increase in numbers of sheaths and large ringlets, there was a marked reduction in the numbers of intact particles and naked inner cores. On the other hand, PCMB treatment did not seem to produce ringlets, but appeared to have a degradative effect upon the sheaths. In addition, these preparations occasionally exhibited long tubular structures several hundred millimicrons in length which differed markedly from the structure of the individual particles. This difference in chemical reactivity of pyocin to urea and to PCMB suggests that the protein content of the sheaths and cores is dissimilar.

Previous studies have indicated that sera from a variety of animals enhanced the activity of colicin (1, 28, 29). Therefore, similar studies were performed with pyocin. Serum was added to samples of purified pyocin to obtain a final concentration of 0.1, 1.0, 5.0, 10.0, and 25.0%. After 1 hr of incubation of the mixtures at 37 C, pyocin activity was measured. Bovine, calf, chicken, and horse sera had no effect on activity, whereas aged (frozen 3 years) human sera enhanced activity threefold at 10.0%, and fivefold at 25%. In addition, aged 25% rabbit serum (frozen 1 year) enhanced pyocin activity fourfold, whereas fresh human serum had no effect on activity. Supplements of either purified hemoglobin (2 to 20 $\mu\text{g/ml}$) or serum albumin (2 to 20 $\mu\text{g/ml}$) did not enhance activity.

DISCUSSION

Despite the fact that bacteriocins have not been thoroughly investigated for their possible role in infectious processes, recent studies with

FIG. 6. Electron micrograph of a purified pyocin preparation stained with uranyl acetate. Segmentation and periodicity of the particles are readily discernible. Long filaments appear to be inner cores distally attached. $\times 320,000$.

P. aeruginosa (13, 14) and *E. coli* (9, 10) indicate that these agents are closely related to classical O endotoxins, or fractions thereof. Consequently, to characterize pyocin comprehensively, it was of interest to determine whether it exerted a toxic effect in vivo. However, the resultant lack of lethality and of dermonecrosis appeared to rule it out as a possible toxic factor in *P. aeruginosa* infections.

At the present time, the in vitro studies require further study and clarification. Although previous studies by Ishii et al. (16) suggested that pyocin may undergo contraction or relaxation, it is not known whether the uncontracted form is responsible for bacteriocinogenic activity. To the present time, we have never been able to observe uncontracted particles in our preparations, regardless of purity. Furthermore, the presence of hollow sheaths in purified pyocin preparations suggests that some particles are either damaged during the purification pro-

cedure or they are incompletely synthesized during induction. At present, it is not known whether the hollow sheaths can also exert antibacterial activity. Since previous studies indicated that freezing and thawing resulted in the contraction of phage T₄ tail protein (30), all of our electron micrographs were obtained with unfrozen preparations.

Although some small round forms or ringlets were detected in both crude and purified preparations intact phage, tail-free heads, or "ghosts" were not observed during this study, nor were plaques produced on pyocin-sensitive strains. Therefore, our findings differ from the observations of Kageyama et al. (20), who noted the presence of an intact, but immunologically distinct, infectious phage fraction which was eluted from DEAE cellulose after the main pyocin peak. The size of the two ringlet forms frequently observed in our preparations appears to correspond quite closely to the diameter of the tail and its

FIG. 7. Electron micrograph of a purified pyocin preparation treated with 8M urea and stained with uranyl acetate. Large ringlets and empty sheaths are numerous whereas intact contractile rod-like particles are infrequently observed. $\times 260,000$.

sheath; their small size, however, rules out the possibility that they are phage heads. Although pyocin has previously been reported to consist entirely of protein (18, 19), it does morphologi-

cally resemble the rod-shaped, single-stranded deoxyribonucleic acid (DNA), headless Pf phage recently isolated from *P. aeruginosa* (26). This phage forms minute plaques on indicator strains

FIG. 8. Electron micrograph of a purified pyocin preparation treated with 10^{-4} M PCMB and stained with uranyl acetate. Inner cores enveloped by fragmented sheath appear to be the result of this treatment. $\times 195,000$.

and resembles pyocin cores in that it appears as hollow rods once it is without its DNA.

At present, the relationship between pyocin and bacteriophage remains unclear; however, the

general appearance and size of the contractile tail-like pyocin forms certainly suggest a close structural similarity to the *E. coli* T₄ phage tail moiety (5), although no base plate, spikes, or

tail fibers were ever observed in these preparations. It has been suggested that pyocin particles may possess such structures (16); however, we were unable to observe these structures in our preparations. In addition, the pyocin structures also resemble those seen in the induced lysates of defective lysogenic strains of *E. coli* (2) and other systems (6, 22, 25, 27). Despite the structural similarities between pyocin and phage, Ikeda et al. (15) showed that a *P. aeruginosa* phage isolated from pyocin lysates was not neutralized by antipyocin sera, thereby suggesting that they may be chemically unrelated. On the other hand, Homma and Suzuki (13) demonstrated that antisera to the protein moiety of *P. aeruginosa* endotoxin neutralized the activity of pyocin from the same organism (strain P1-III). Therefore, it is primarily this problem of relatedness between the two systems that requires further elucidation.

Another problem which remains to be resolved is the mechanism by which pyocin activity is rapidly lost upon freezing and thawing, although normal refrigeration and even room temperature maintain the antibacterial activity. Despite the striking loss in activity, electron micrographs did not demonstrate discernible morphological changes of the particles after freezing and thawing. Nevertheless, these results appear to be consistent with those of Kageyama (19), who noted a loss in activity upon lyophilization of pyocin. One can assume that the stability of crude lysates to freezing is due to the presence of protective impurities which are subsequently removed during the purification procedure. On the other hand, the comparatively slight enhancement of activity by aged human and rabbit sera, but not by human hemoglobin or serum albumin, suggests that pyocin differs in its mode of action from the colicine K system (1, 28, 29) which was stimulated several hundred-fold by these serum supplements.

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