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Defective Pyocin Particles Produced by Some Mutant Strains of *Pseudomonas aeruginosa*

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Mutants of *Pseudomonas aeruginosa*, defective in the production of active R-type pyocins, were isolated from pyocinogenic strains and their products were characterized. Polysheath-like structures were found in induced lysates of 29 out of 42 mutants. Two mutants (strain P15-16 and M189) were found to produce special defective particles, which were characterized in detail. The other 11 mutants did not produce significant amounts of any structure visible under an electron microscope. Serum blocking powers were found in lysates from P15-16 and M189 to significant amounts. Defective particle produced by strain P15-16 lacked the sheath component, whereas M189 had morphological defects at the junction between sheath and baseplate, and also in the architecture of baseplate. Both defective particles could adsorb to the surface of bacteria, that were sensitive to pyocin, at the tip of their fibers without killing cells. All M189 particles attached to the bacteria had the extended sheaths. Therefore, attachment to the bacteria by fibers is not sufficient to kill cells, and contraction of sheath must occur after the initial adsorption by fibers for pyocin to express its biological activity. Defective particles of strain P15-16, which was derived from strain P15 (a pyocin R1 producer), could be converted to active forms by an in vitro complementation reaction with extracts from certain mutants originated from strain PAO (a pyocin R2 producer). This result indicated the exchangeability of components between R-type pyocins belonging to the different groups.

R-type pyocins, a group of bacteriophage tail-like bacteriocins, have been found in many strains in *Pseudomonas aeruginosa* (4, 8, 9). They are classified into four groups (R1, R2, R3, and R4) according to the difference in their receptor specificity, but they are morphologically indistinguishable from each other and are also very similar in their immunological properties (8).

The R-type pyocins have a structure resembling the phage tail with a contractile sheath of T-even coliphages (6, 8). Cessation of the synthesis of macromolecules was shown to occur in the sensitive cells after pyocin adsorption (14). The pyocin particles adsorbed to the sensitive cells or to the isolated membrane fragments have the contracted sheath and attached to the receptor site at the tip of core tube (5).

To understand the relation between the pyocin structure and its bactericidal activity, we examined the products that were produced by pyocin-less mutants isolated from pyocinogenic strains. Two mutants were found to produce special defective particles and these defective particles were characterized in detail. Both defective particles could adsorb to the sensitive cells by their fibers but did not kill them. The lack of lethal effect is probably due to a defect of the sheath component in one case and a defect of sheath contraction in another. It was concluded from these results that, in the absence of contraction of the sheath, adsorption of pyocin particles to sensitive cells by their fibers was not sufficient to manifest their bactericidal action.

We also present evidence that supports the close relationship among different groups of R-type pyocins. One of the defective particles was found to be converted to an active form by an in vitro complementation reaction with extracts from certain mutants isolated from the strain pyocinogenic for a different group of R-type pyocin.

MATERIALS AND METHODS

Bacterial strains. Pyocinogenic strains, indicator strains, and some of the pyocin mutants used in this study are listed in Table 1. Other pyocin mutants are in Table 2. Pyocin mutants were independently isolated by the method described previously (10) from P15, P28, and derivatives of PAO after mutagenesis with *N*-methyl-*N'*-nitrosoguanidine.

Media and buffers. Nutrient broth (NB), nutrient

TABLE 1. *Bacterial strains used*

Strain	Character	Origin	Reference
Pyocinogenic strains			
P15	Pyocin R1+[a]		12
M11	*trp*, *str*, pyocin R2+	PAO	8
M12	*trp*, *cml*, pyocin R2+	PAO	10
M28	Pyocin R4+		8
Pyocin mutants[b]			
P15-16	*trp*, *str*, pyocin R1-	P15	8
M188	*trp*, *his*, *ilv*, *cml*, pyocin R2-	PAO	
M189	*trp*, *his*, *ilv*, *cml*, pyocin R2-	PAO	
Indicators			
P14	R1[s], R2[s], R4[s]		12
P14d	R1[s], R2[r]	P14	12
P14f	R1[r], R2[s]	P14	12

[a] Pyocin productivity. For example, pyocin R1+ denotes the production of pyocin R1, and pyocin R1- signifies that the mutant is derived from a pyocin R1-producing strain but does not produce active pyocin R1.

[b] Pyocin mutants used frequently in this paper are listed. The other pyocin mutants are shown in Table 2.

TABLE 2. *Classification of pyocin mutants according to the major structures observed in the MC lysates*[a]

Structure	Strain[b]	No.
Polysheath[c]	M47, M106, M107, M108, M111, M113, M114, M185, M186, M187, M188, M191, M192, M193, M195, M196, M197, M202, M203, M204, M205, M206, M207, P28-1, P28-2, P28-7, P28-8, P28-10, P28-12	29
P15-16 particle[d]	P15-16	1
M189 particle[e]	M189	1
None	M110, M199, M200, P28-3, P28-5, P28-6, P28-9, P28-11, P28-14, P28-15, P28-16	11

[a] Electron microscopic observations of the lysates were performed as described in the text. Mutants were classified into four groups according to the major structures observed. Small amounts of apparently complete structures, contracted sheath, or free core tubes observed in certain mutant lysates were ignored to construct the table.

[b] P15-16 was isolated from P15 (pyocin R1+). Strains prefixed with a letter M were all derived from strain PAO (pyocin R2+) and the other strains were derivatives of P28 (pyocin R4+).

[c] Mutants which can produce polysheath as a major component are included in this class.

[d] The concentration of P15-16 particles in P15-16 lysate was approximately one-fifth of that of pyocin particles in P15 lysate.

[e] The concentration of M189 particles in M189 lysate was usually less than one-fifth of that of pyocin particles in M11 lysate.

plating agar, and synthetic G medium were prepared as described previously (18). Liquid cultures were grown in NB or G medium supplemented with required amino acids at 37 C with shaking. Amino acids were added in a concentration of 100 μg/ml and vitamin-free Casamino Acids (Difco) were used in a concentration of 1 mg/ml. Dilution buffer (DB) used to dilute pyocin solutions was 0.085 M NaCl-0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6)-1 mM $MgCl_2$. Saline buffer was 0.15 M NaCl-0.01 M Tris-hydrochloride buffer, pH 7.5. SDS-SH buffer was 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol in 0.0625 M Tris-hydrochloride, pH 6.8.

Mitomycin C lysate. Mitomycin C (MC) lysates were prepared from pyocinogenic cells and from mutant cells according to the method of Kageyama (9). Liquid cultures were grown in NB or supplemented G medium to a late logarithmic phase (about 3×10^8 cells/ml) and treated with MC at a concentration of 1.5 μg/ml. Growth was followed by turbidometry. Spontaneously lysed cultures were treated with a few drops of chloroform and deoxyribonuclease (EC 3.1.4.5) (10 μg/ml). Some mutant cultures did not lyse spontaneously, but could be lysed artificially by shaking in the presence of chloroform. Unless otherwise indicated, pyocin activity was measured by a serial dilution method (9) using strain P14 as an indicator.

Preparation of concentrated extract for in vitro complementation reactions. Magnesium sulfate was added to the culture medium in a final concentration of 20 mM to delay the onset of spontaneous lysis of MC-induced cultures. The induced cultures (150 ml) were centrifuged 2 h after induction. The collected cells were lysed by incubating them with 0.5 ml of egg white lysozyme (EC 3.2.1.17) (0.5 mg/ml)-ethylenediaminetetraacetic acid (40 mM)-0.01 M Tris-hydrochloride (pH 7.5) at 30 C for 15 min. The viscous extracts were occasionally stirred with a glass rod during incubation. Then the extracts were treated with 0.5 ml of deoxyribonuclease (0.2 mg/ml)-$MgSO_4$ (80 mM)-0.01 Tris-hydrochloride (pH 7.5). Incubation was performed at 30 C to complete reduction of viscosity. The concentrated extracts were used for in vitro complementation reactions on the same day or

stored frozen at −20 C. The frozen extracts were thawed immediately before the experiment.

Preparation of pyocin particles. Pyocin R1 was purified according to the method of Yui (25).

Radioactively labeled [^{14}C]pyocin R1 and R2 were prepared by the method reported previously (20). P15 cells were grown in G medium and M12 cells were in G medium supplemented with tryptophan. The induced cultures were labeled with ^{14}C-labeled amino acid mixture (0.67 Ci/g) added three times at 40, 60, and 80 min after induction at a concentration of 5 μCi/ml. Lysates were centrifuged at 10,000 rpm for 10 min to remove debris and fractionated by sucrose gradient centrifugation. Pyocin fractions were detected by measuring the radioactivities of a portion of each fraction in Triton-toluene scintillator. The peak fractions were pooled and dialyzed against saline buffer.

Preparation of defective pyocin particles. Defective pyocin particles produced by P15-16 and M189 were partially purified by the following method. MC lysate (150 ml) was prepared from the culture grown in G medium supplemented with Casamino Acids and tryptophan. The lysate was centrifuged to remove debris, and the supernatant was centrifuged at 42,000 rpm for 3 h in a Hitachi RP42 rotor. The pellet was gently dissolved in 1.5 ml of saline buffer. The solution was centrifuged at low speed, and then the supernatant was fractionated by 5 to 20% (wt/wt) linear sucrose gradient made in saline buffer. Centrifugation was performed at 25,000 rpm for 7 h in a Hitachi RPS25 swinging rotor. Defective particles of P15-16 were detected by serum blocking assay and with an electron microscope. Defective particles of M189 were detected by a peak in an absorbancy at 280 nm and also with an electron microscope. The peak fractions (see fractions 9, 10, and 11 in Fig. 3a, and fractions 18 and 19 in Fig. 3b) were pooled and dialyzed against saline buffer.

^{14}C-labeled defective particles were prepared as follows. Cultures of P15-16 and M189 were grown in G medium supplemented with requiring amino acids. Ten milliliters of the induced culture was labeled with 20 μCi of ^{14}C-labeled amino acid mixture (0.67 Ci/g) at 40, 60, and 80 min after induction. The lysate prepared at 3 h after induction was centrifuged at 50,000 rpm for 100 min in a Hitachi RP65T rotor. The pellet was dissolved in 1 ml of saline buffer and centrifuged in a sucrose gradient as described above. The radioactivities precipitable with the anti-pyocin serum were measured. Peak fractions were pooled and dialyzed against saline buffer.

Electron microscopy. The morphological survey of MC lysates prepared from pyocin mutants was carried out by the agar filtration method described by Kellenberger and Arber (15). Definite amounts of polystyrene latex spheres were added to the lysates to facilitate the quantitative calculation of the concentration of structures observed in an electron microscope.

All specimens were negatively stained with 1% neutralized phosphotungustic acid (1) and examined under a JEOL 100B electron microscope at 80 kV.

Serological method. Antisera against pyocin R1 and pyocin R2 were prepared as described previously (21). Antiserum against contracted sheath isolated from pyocin R1 had been prepared by Yui (25). The serum blocking assay was performed by one of the following two methods. (Method I) A sample (0.2 ml) was mixed with 0.2 ml of antiserum diluted in NB to a first-order rate constant of neutralization (k) of about 0.2 per min. The mixture was incubated overnight at 37 C. The residual neutralizing activity was then measured by the inactivation of subsequently added tester pyocin as follows. A portion (20 μl) of the mixture was added to 80 μl of P15 (pyocin R1$^+$) or M11 (pyocin R2$^+$) lysate diluted with NB to give the pyocin activity of about 5×10^8 U/ml. After a further 1 h of incubation at 37 C, the tubes were titrated for the remaining pyocin activity. (Method II) Sample solution (25 μl) was mixed with 25 μl of antiserum diluted in DB to a k value of about 1 per min. After overnight incubation at 37 C, 50 μl of P15 or M11 lysate (about 10^4 U/ml) was added to the mixture as a tester. At the end of incubation for 1 h at 37 C, 1 ml of DB was added to the tubes to stop the reaction, and the remaining pyocin activity was assayed. Serum blocking powers were presented as a percentage of serum blocked $(1-k_{final}/k_{initial}) \times 100$.

Immunoprecipitation reactions were performed according to the method described previously (20).

In vitro complementation reaction. Equal volumes (25 μl each) of two concentrated extracts prepared from the induced cultures of mutants were mixed and incubated at 30 C for the time indicated. At the end of incubation, 1 ml of DB was added to stop the reaction, and pyocin activity was assayed. Complementation reactions using MC lysate were carried out in the same way.

SDS polyacrylamide slab gel electrophresis. Samples for SDS polyacrylamide gel electrophoresis were prepared according to the method reported previously (19, 20). The ^{14}C-labeled pyocin and ^{14}C-labeled defective particles were specifically precipitated by anti-pyocin serum with carrier pyocin. The precipitates were washed twice with saline buffer and finally with acetone and were dissolved in 50 μl of SDS-SH buffer (20). Dissociation to the subunits was accomplished by immersing the tubes for 5 min in boiling water.

SDS polyacrylamide slab gel electrophoresis was carried out according to the SDS discontinuous buffer system described by Maizel (17) and Laemmli (16). The gels had an acrylamide:methylene bis acrylamide ratio of 30:0.8, and polymerization was initiated by adding small amounts of *N,N,N',N'*-tetraethylmethylenediamine and ammonium persulfate. The resolving and spacer gels contained 15 and 5% acrylamide. The width of the gel slab was 1.5 mm. Electrophoresis was carried out using Hoeffer Scientific Instruments model SE-500 apparatus for 4 h at 60 mA. Proteins were stained by incubating the gel slab for 1 h at 37 C in 0.1% Coomassie brilliant blue freshly dissolved in 25% isopropanol-10% acetic acid and destained by a diffusion method in 10% acetic acid.

The stained gel slab was dried according to the method described by Maizel (17). Sakura X-ray film (Medical, New Y type) was used to take autoradiograms.

Chemicals. MC was purchased from Kyowa Hakko Kogyo Co., Ltd. Deoxyribonuclease I was a product of

Miles-Seravac Ltd. and egg white lysozyme was a product of Seikagaku Kogyo Co., Ltd. ^{14}C-labeled amino acid mixture was purchased from New England Nuclear. Acrylamide and methylene-bis-acrylamide were purchased from Seikagaku Kogyo Co., Ltd. Tetramethylenediamine was a product of Tokyo Kasei Kogyo Co., Ltd. SDS was purchased from Merck. Coomassie brilliant blue R-250 was purchased from Nakarai Chemicals Company. Polystyrene latex sphere (0.087 μm) was purchased from Dow Chemicals Company. Other chemicals were of chemical grade.

RESULTS

General characterization of pyocin mutants. MC lysates of pyocin mutants were first examined morphologically, and the results are summarized in Table 2. In this table, mutants are classified according to the major structures observed in the MC lysates under an electron microscope.

Polysheath-like structures, as shown in Fig. 1a, were observed in many lysates. They were abnormally long and have a width of contracted sheath of pyocin. They could bind with antibodies against sheath isolated from pyocin R1. These aberrant structures were often observed in a form of large aggregate (Fig. 1a).

Strains P15-16 and M189 were found to produce certain defective particles. Photographs of the lysates concentrated by ultracentrifugation are shown in Fig. 1b and c. P15-16 produced defective particles composed of a core tube, baseplate, and fibers. Defective particles produced by M189 were hardly distinguishable from active pyocin particles in their morphology. But it is noticeable that particles with contracted sheath or free contracted sheaths could not be observed in M189 lysate. Properties of these defective particles are reported in detail in the following sections.

Pyocin particles, free contracted sheath, and naked core tube were observed in some mutant lysates, but in very small amounts. Other structures, such as free fibers or baseplates, were not visible in an electron microscope.

Serological characterization of mutant lysates were carried out by serum blocking assay. The serum blocking assay detects those antibodies which inactivate pyocin on complexing with it. Serial dilutions of lysates were added to anti-pyocin serum, and the k values of the residual serum were measured after the reaction went to completion. Quantitative and qualitative differences in the antigen composition in the lysates can be assayed by this method (2). Most of the mutant lysates studied did not show significant amounts of serum blocking antigens. The concentrations of these mutant lysates which showed 50% serum blocking powers were at least 100-fold higher than that of the lysates of pyocinogenic strains. Lysates prepared from P15-16 (pyocin R1$^-$) and M189 (pyocin R2$^-$) were found to have reduced, but significant, amounts of serum blocking antigens (Fig. 2). P15-16 and P15 lysates were incubated with anti-pyocin R1 serum or anti-sheath serum (Fig. 2). P15-16 lysate did not show any blocking power to anti-sheath serum, as expected from the result that P15-16 cells did not produce any visible structure related to sheath. Figure 2a also shows that most, if not all, neutralizing antibodies in the anti-pyocin R1 serum were blocked by P15-16 lysate. The antisheath antibodies remaining after absorption of the anti-pyocin R1 serum by P15-16 lysate should be active in neutralization of tester pyocin, but they were much less effective than the antibodies against the other components, such as fibers, and the reaction rate was so slow as not to be detected under the conditions used. M189 lysate was also shown to contain most, if not all, antigen species but in a reduced concentration relative to M11 (pyocin R2$^+$) lysate (Fig. 2b).

Several of the mutants examined in the above experiments had been employed to map the genetic loci of pyocin genes (R1, R2, and R3) on the chromosome of *P. aeruginosa* (10-13), but a fine genetic analysis of these pyocin mutants has been difficult so far.

Structure of defective particles produced by P15-16 and M189. Defective particles produced by P15-16 and M189 were partially purified as described above. The sucrose gradient profiles are shown in Fig. 3. P15-16 particle (defective particle of P15-16) was identified by serum blocking assay and electron microscopy, and identification of M189 particle (defective particle of M189) was by electron microscopy only. The sedimentation velocity constant was calculated to be approximately 45S for P15-16 particle (Fig. 3a) and 90S for M189 particle (Fig. 3b) from the position of pyocin R1 (90S) indicated by arrows. Electron micrographs of the peak fractions are shown in Fig. 4a and b. Enlarged photographs of the representative particles are shown in Fig. 4c and d. Note that both defective particles have fibers and that the sheath of M189 particle is not contiguous to the baseplate. The architecture of baseplate in M189 particles is somewhat different from pyocin R1 particle (Fig. 4e).

Serum blocking powers of partially purified defective particles were compared to those of purified pyocin particles. The results, shown in Fig. 5, indicated that the isolated P15-16 and

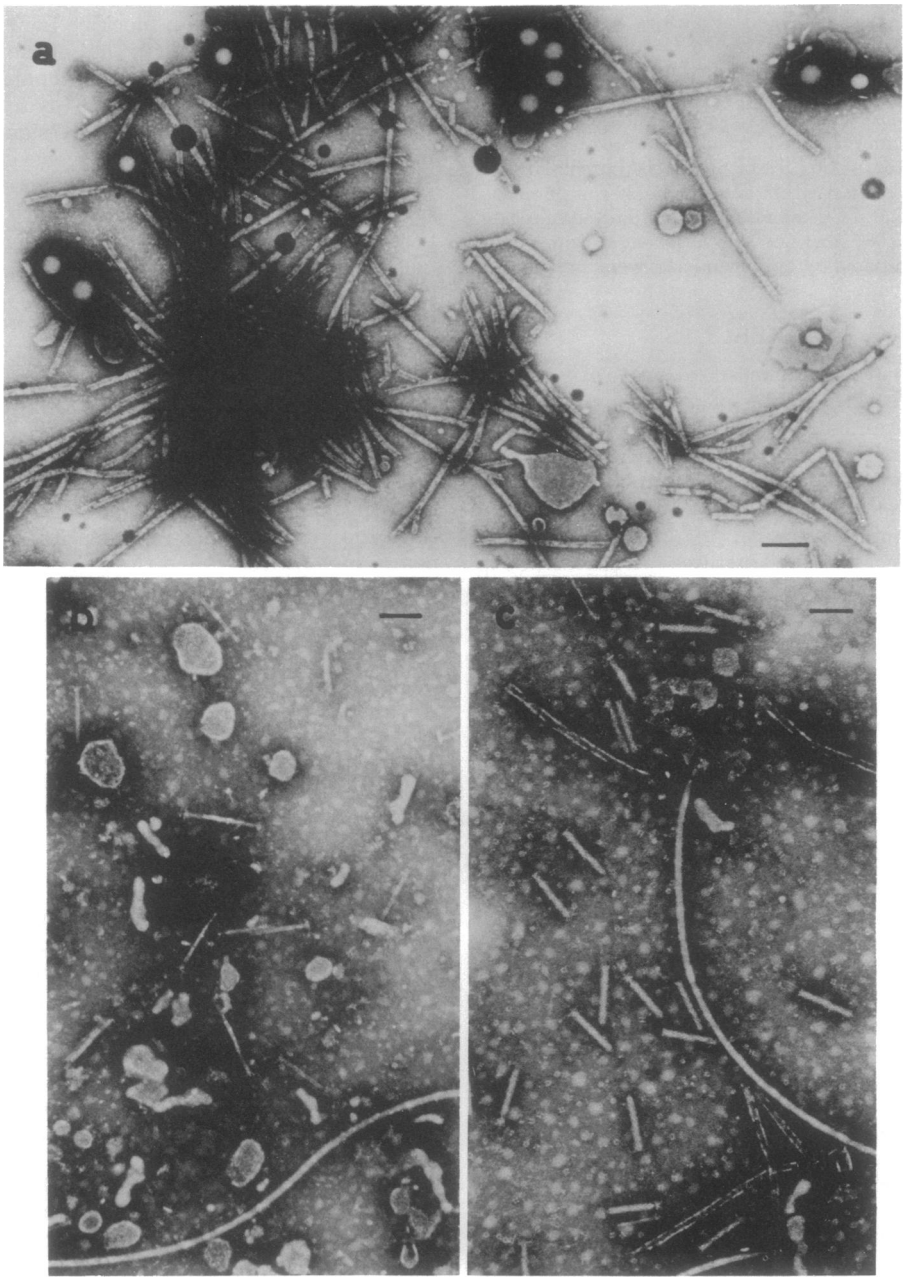

FIG. 1. *Electron micrographs of mutant lysates. (a) M206 lysate. Many polysheath-like structures are seen. (b) P15-16 lysate concentrated by ultracentrifugation. The lysate was centrifuged at 60,000 rpm for 2 h. The pellet was dissolved in 1/10 of the original volume of saline buffer. Defective particles lacking sheath component are seen. (c) M189 lysate concentrated by ultracentrifugation as described above. Particles similar to active pyocin and polysheath-like structures are seen. The bars in these and in the subsequent micrographs represent 100 nm.*

M189 particles contained most, if not all, of serum blocking antigens presented in pyocin R1 and R2, respectively.

Subunit proteins in these defective particles were determined (Fig. 6). Labeled defective particles, prepared as described above, were subjected to SDS polyacrylamide slab gel electrophoresis. Labeled pyocin R1 and pyocin R2

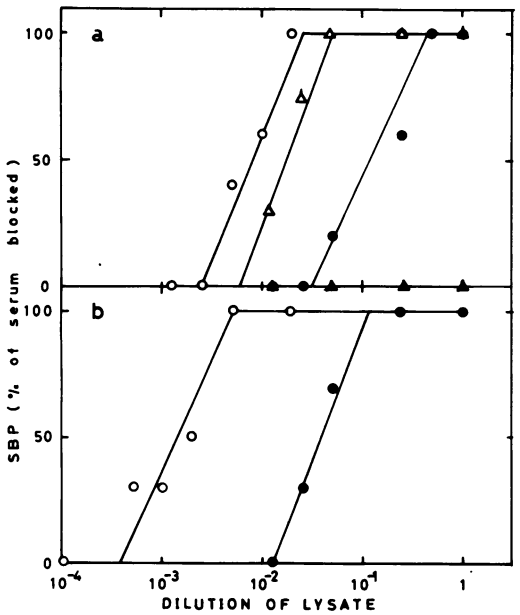

FIG. 2. *Blocking of anti-pyocin serum by mutant lysates. SBP were measured by the method I described in the text. (a) Blocking of anti-pyocin R1 serum (O, ●) and anti-R1 sheath serum (Δ, ▲) by P15 lysate (open symbols) and P15–16 lysate (closed symbols). (b) Blocking of anti-pyocin R2 serum by M11 lysate (O) and by M189 lysate (●). SBP, Serum blocking powers.*

were used as references. Bands are numbered according to the previous report (19). P15–16 particle (Fig. 6a) was found to be lacking bands no. 6, 7, 11, and 13, and probably no. 16b, all of which corresponded to the subunit proteins present in isolated sheaths (19). In M189 particle (Fig. 6b), two bands (no. 11 and 13) were missing and the position of band no. 6 (the major subunit in sheath) shifted to a little lower molecular weight (compare with pyocin R2 [Fig. 6e]).

Adsorption of defective particles to bacteria. P15–16 and M189 particles were shown to have fibers as described above. Adsorption of these particles to the cells sensitive to pyocin and to the envelope fragments released from them was demonstrated with an electron microscope (Fig. 7 and 8). Figure 7a shows that P15–16 particles attach to the surface of the cell at the tip of their fibers. In Fig. 7b, P15-16 particles attached to the fragments probably released from cell envelope are shown. Figure 8a and b show M189 particles adsorbed to the surface of a whole cell and to an envelope fragment by their fibers. Note that M189 particles attached to the cell or to an envelope fragment have the extended sheaths. Active pyocin particles adsorbed to the sensitive cells or envelope fragments have the contracted sheaths and attached to the cell surface at the exposed core tubes, as shown by Ikeda and Nishi (5) and in Fig. 8c. The binding was specific for sensitive cells. The defective particles did not adsorb to the corresponding resistant bacteria, i.e., P14f for P15–16 particles and P14d for M189 particles.

In vitro conversion of defective particles to active forms. In vitro complementation tests were performed using concentrated extracts prepared from induced cells of various pyocin mutants. Incubation of P15–16 extract with the extracts prepared from certain other mutants resulted in the formation of active pyocin, as shown in Table 3. MC lysate of P15–16 was also effective in the complementation reaction, but the partner extracts could not be replaced by

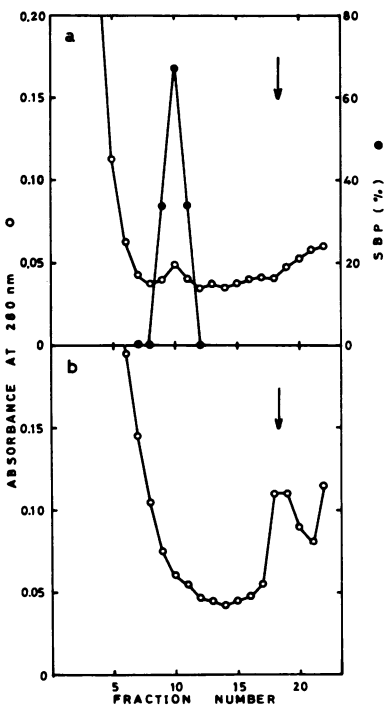

FIG. 3. *Isolation of defective particles from P15–16 and M189 lysates by centrifugation in sucrose gradient. Defective particles produced by P15–16 and M189 were partially purified from the MC lysates according to the method described in the text. This figure shows the sucrose gradient profiles. Serum blocking power (SBP) was measured by the method II for serum blocking assays. The peak fraction of pyocin R1 centrifuged in the same condition is indicated by an arrow which corresponds to 90S. (a) P15–16, (b) M189.*

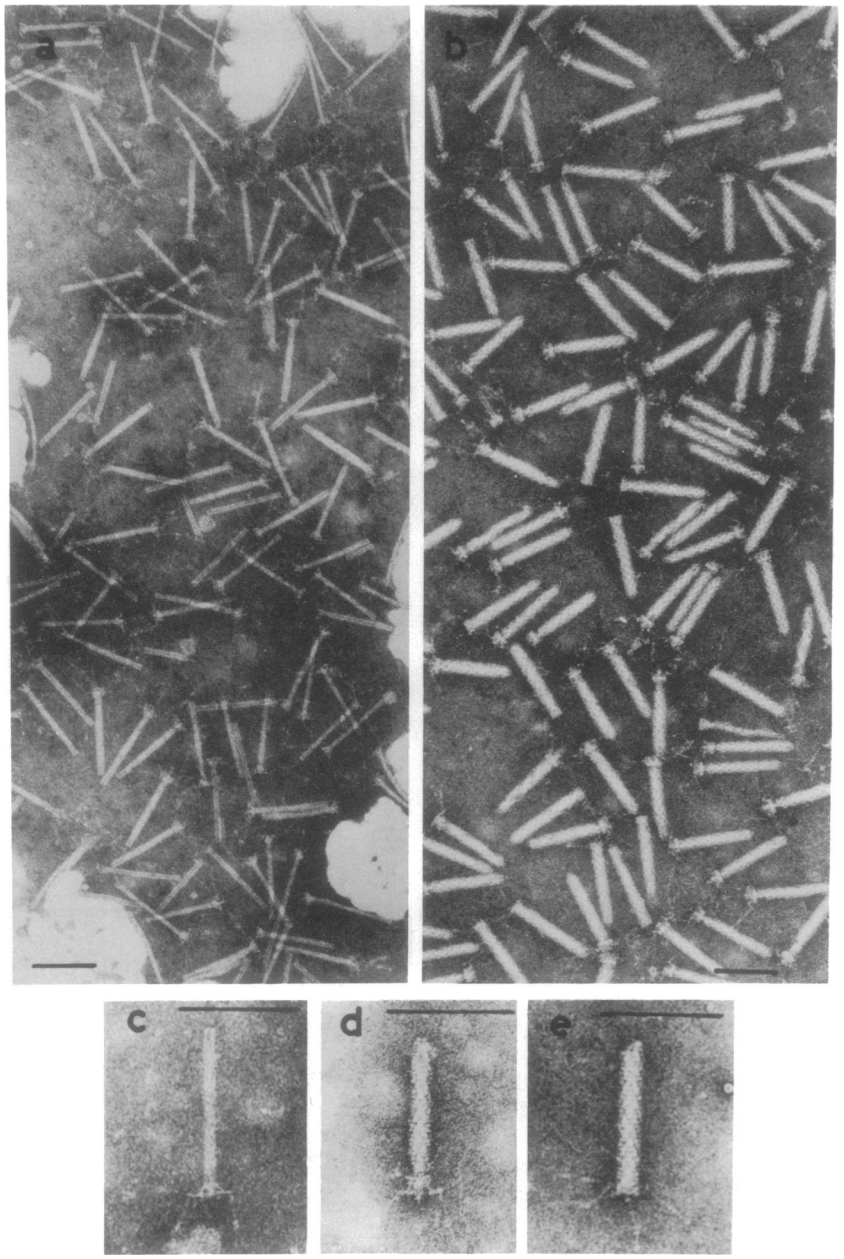

FIG. 4. *Electron micrographs of defective pyocin particles. (a) Partially purified P15-16 particles. (b) Partially purified M189 particles. (c) P15-16 particle. (d) M189 particle. (e) Pyocin R1 particle.*

the corresponding MC lysates. Complementation with other combinations of the mutants was unsuccessful.

As shown above, P15-16 produced defective particles lacking the sheath component, and no structures related to sheath were found in the lysate. The following features of the complementation reaction support the model that assemblage of sheath subunit(s) around the core tube of P15-16 particles results in the formation of active pyocin particles.

(i) All the mutants which could complement in vitro with P15-16 were those which produced polysheath (Table 2).

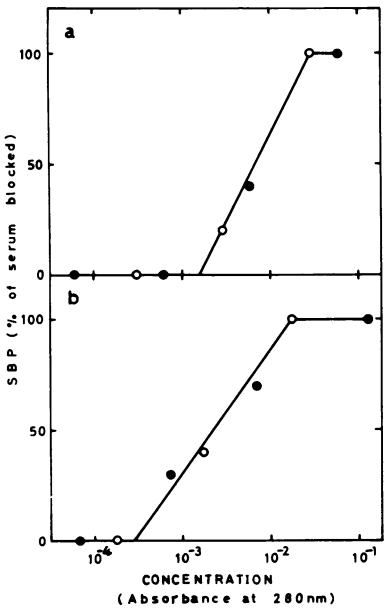

FIG. 5. *Blocking of anti-pyocin serum by partially purified P15-16 and M189 particles and purified pyocin particles. Serum blocking powers (SBP) were measured by the method II described in the text. Concentrations of particles are expressed as absorbance at 280 nm of the sample solution used. (a) Blocking of anti-pyocin R1 serum by pyocin R1* (○) *and P15-16 particles* (●). *(b) Blocking of anti-pyocin R2 serum by pyocin R2* (○) *and M189 particles* (●).

(ii) The action spectrum of killing substances that appeared as a consequence of the complementation reaction between extracts of P15-16 (pyocin R1$^-$) and pyocin R2$^-$ mutants was of pyocin R1 type (Table 4).

(iii) The time course of the reaction was examined in various concentration of the reactants. The results are shown in Fig. 9. When the variously diluted P15-16 lysate was incubated with M188 extract, the reaction reached a plateau within 5 min irrespective of the concentration of P15-16 lysate, and the pyocin activity that appeared was proportional to the concentration of P15-16 lysate (Fig. 9a). In contrast, when M188 extract was decreased in the reaction mixture, a drastic reduction in the rate of reaction was caused (Fig. 9b). As pyocin activity did not increase by further incubation beyond 60 min, the final level was not proportional to the concentration of M188 extract. The results are consistent with the hypothesis that one P15-16 particle is converted to one active pyocin particle by assemblage of large numbers of sheath proteins on it.

(iv) The pyocin activity appeared as a consequence of the complementation reaction had the same sedimentation constant (90*S*) as that of native R-type pyocin particles, when examined by sucrose density gradient centrifugation.

(v) Finally, conversion of P15-16 particles to pyocin particles was demonstrated by subunit analysis. Radioactively labeled P15-16 lysate was incubated with unlabeled M188 extract, and then assembled pyocin particles (90*S*) were separated from P15-16 particles (45*S*) by sucrose gradient centrifugation. The isolated particles were precipitated with anti-pyocin R1 serum, dissociated by heating in SDS, and subjected to SDS gel electrophoresis. The radioactive proteins were located by the autoradiogram of the gel (Fig. 6c). All the protein species composing P15-16 particles (Fig. 6a) and no other protein species of pyocin were radioactive in the assembled pyocin particles. Several minor bands not corresponding to pyocin proteins would be non-pyocin proteins contaminating in the 90*S* fraction.

DISCUSSION

Structure and function of R-type pyocins. R-type pyocins are defined as a class of bacteriophage tail-like bacteriocins which cross-react serologically with pyocin R1 (so far referred to as pyocin R) and have been found in many strains of *P. aeruginosa* (8). These pyocins are classified into several groups according to the action spectrum (8). Results obtained from morphological, biochemical, immunological, physiological, and genetical studies on R-type pyocins (7, 8, 10-13) consistently support a hypothesis that these pyocins belong to the closely related entities probably arisen from a common ancestor. We will here discuss some features on the relationship between the structure and function of R-type pyocins.

The fine structure of pyocin R1 was first reported by Ishii et al. (6). On their report, the main structure of pyocin R1 is a double hollow cylinder, 120 nm long and 15 nm in outer diameter, which consists of a contractile sheath and a core tube, and the presence of a baseplate and fibers was also suggested. The structure of the baseplate and fibers, however, had not been well defined. The well-imaged electron micrographs of pyocin R1 and two defective pyocin particles allowed us to describe more detailed structure of these components (Fig. 4). Fibers are approximately 40 nm in length and 2 nm in diameter and have no kink. The presence of a small knob at the tip and of two small ball-like

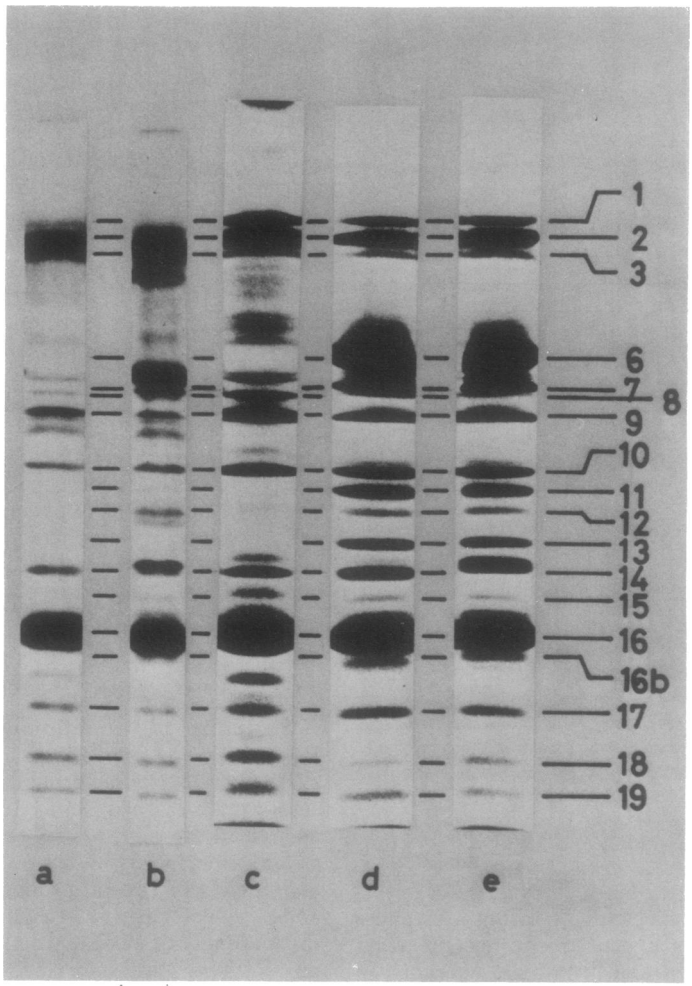

FIG. 6. *Protein composition of defective particles. ^{14}C-labeled P15-16 and M189 particles and labeled pyocin particles isolated by sucrose gradient centrifugation were precipitated with anti-pyocin serum. The precipitates were dissolved in SDS-SH buffer, boiled, and applied to 15% polyacrylamide slab gel. Autoradiograms were taken. (a) P15-16 particles; input radioactivities, 7.5 × 10^3 dpm/gel. (b) M189 particles; 2.2 × 10^4 dpm/gel. (c) Assembled pyocin particles isolated after incubating ^{14}C-labeled P15-16 lysate with unlabeled M188 extract; 6.5 × 10^4 dpm/gel. (d) Pyocin R1 particles; 4.3 × 10^4 dpm/gel. (e) Pyocin R2 particles; 6.0 × 10^4 dpm/gel. Numbers indicated on the right are band numbers assigned according to the previous report (19).*

structures approximately 15 nm apart from the distal end of the fiber was suggested. Baseplates of P15-16 and M189 particles (Fig. 4c and d) can be defined as a structure approximately 10 nm in height and 15 nm in diameter, and it seems to have a thin endplate of approximately 25 nm in diameter, to which fibers are attached. In complete pyocin particles, however, the baseplate could not be resolved as such (Fig. 4e) and had been considered to be a part of sheath.

We found two types of defective pyocin particles produced by mutant strains, P15-16 and M189. That P15-16 particle lacks the sheath component was demonstrated morphologically, serologically, and biochemically. The particle was missing probably all protein species (at least four species) of sheath component. M189 particle is composed of sheath, core tube, baseplate, and fibers. The morphological defect was observed at the junction between sheath and baseplate and also in the architecture of baseplate: the sheath is not contiguous to the baseplate and the baseplate has a less packed structure than pyocin particle (compare Fig. 4d and e). Two species of subunits are missing from the particle, and alteration in size of the major sheath protein was suggested.

Adsorption of these defective particles to the

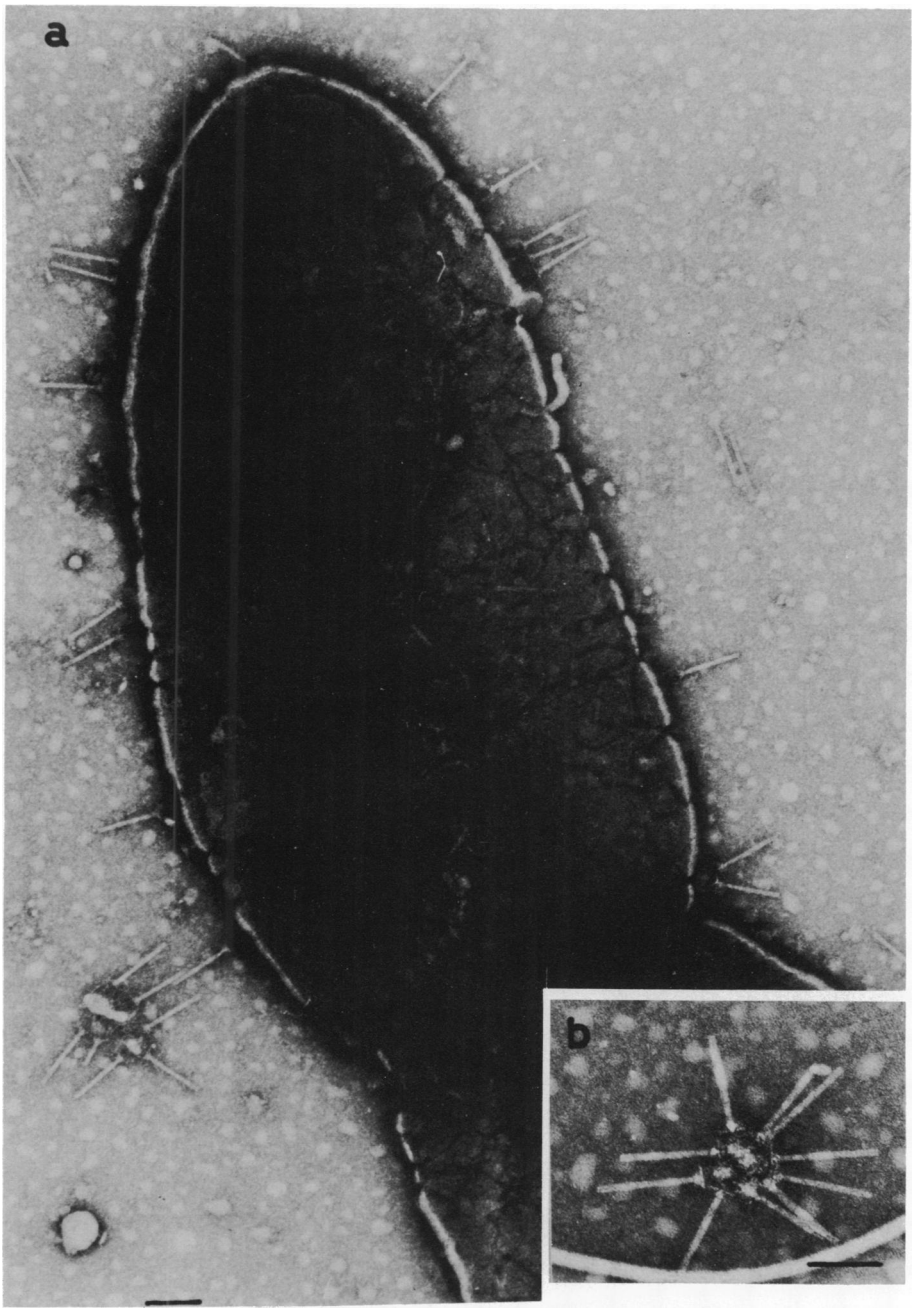

FIG. 7. *Adsorption of P15–16 particles to P14 cell. Partially purified P15–16 particles were incubated for 10 min at 37 C with P14 cells disrupted by freezing and thawing (approximately 100 particles per cell). The specimen was negatively stained. (a) P15–16 particles attached to a P14 cell. (b) P15–16 particles attached to a small fragment probably released from P14 cell envelope.*

surface of the bacteria sensitive to pyocin was demonstrated (Fig. 7 and 8). Attachment of these particles to the cell is effected by the distal tip of their fibers. On the other hand, active pyocin particles adsorbed to the sensitive bacteria attached to the cell surface by their core tubes (5; Fig. 8c). M189 particles do not contract their sheath after attaching to the cells

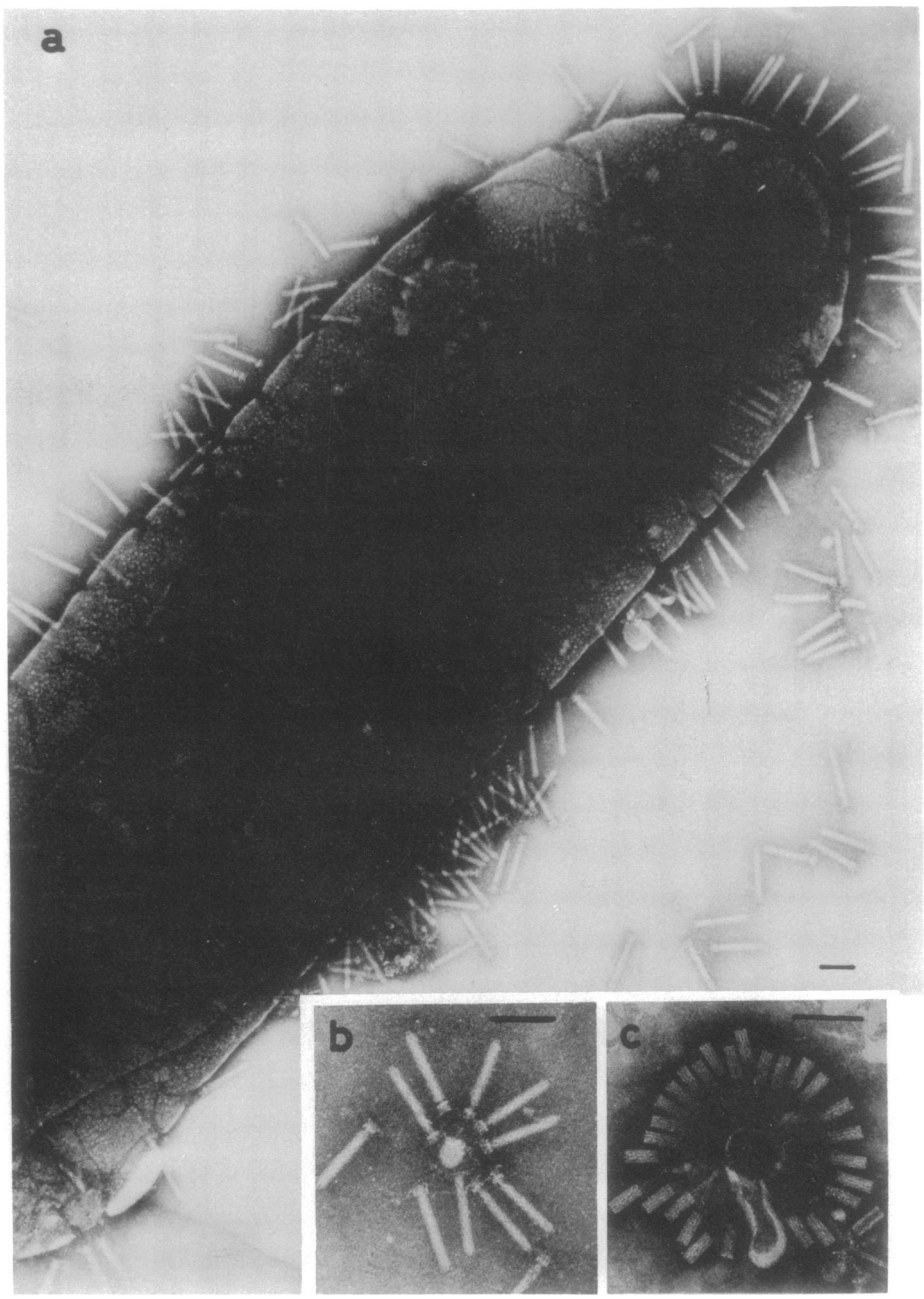

FIG. 8. *Adsorption of M189 particles to P14 bacteria. Partially purified M189 particles were incubated with P14 cells as described in the legend for Fig. 7 (approximately 100 particles per cell). (a) M189 particles attached to a P14 cell. (b) M189 particles attached to a small envelope fragment. In micrograph (c), pyocin R1 particles attached to an envelope fragment from P14 cell are shown.*

(Fig. 8a and b). It is probably due to the defect of the interaction between baseplate and sheath. P15-16 particles could be converted to active pyocin particles by the assemblage of sheath proteins with them. From these results it can be concluded that: (i) the presence of fibers are sufficient for pyocin to attach to the sensitive bacteria, and fibers must be the apparatus

TABLE 3. *In vitro complementation of P15-16 extract*[a]

Extract	Pyocin activity appeared after incubation with:	
	Self	P15-16 extract
None		10
M47	10	800
M106	<10	250
M107	<10	1,300
M188	10	1,300
M189	<10	<10
M193	<10	<10
M196	<10	800
M204	<10	30
P28-2	50	50
P28-7	<10	10
P28-12	30	10

[a] The extract of P15-16 was incubated with the extract from the mutant indicated overnight at 30 C. Pyocin activities are expressed as units per milliliter of the reaction mixture.

TABLE 4. *Action spectrum of pyocin activity appeared as a consequence of complementation reaction*[a]

Extracts incubated	Pyocin activity on		
	P14[b]	P14d[b]	P14f[b]
P15-16 + M47	250	150	<10
P15-16 + M106	500	250	<10
P15-16 + M107	500	250	<10
P15-16 + M188	800	500	<10
P15-16 + M196	500	500	<10

[a] Pyocin activities appeared after incubating the pairwise extracts, as described in Table 3, were titrated on different indicators. Pyocin activities are expressed as units per milliliter of the reaction mixture.

[b] Sensitivities of indicators are as follows. Pyocin R1: P14, sensitive (s); P14d, s; P14f, resistant (r). Pyocin R2: P14, s; P14d, r; P14f, s.

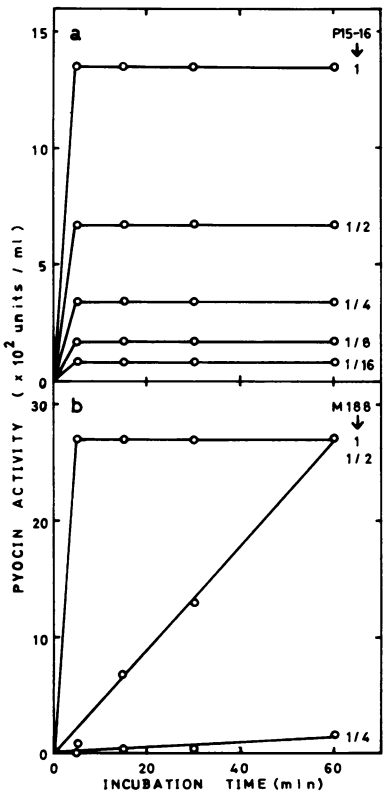

FIG. 9. *Kinetics of the complementation reaction between P15-16 lysate and M188 extract. Incubation was carried out at 30 C for the periods indicated and the reaction was stopped by adding DB. Pyocin activity is expressed as units per milliliter of the reaction mixture. Pyocin activities in P15-16 lysate and M188 extract used in the experiments were negligible. (a) M188 extract was incubated with P15-16 lysate diluted with G medium as indicated by figures. (b) P15-16 lysate was incubated with M188 extract diluted with 0.01 M Tris buffer (pH 7.5) as indicated by figures.*

for the initial attachment of pyocin particles to the receptor sites on the cell; but that (ii) the attachment with fibers is not sufficient and the contraction of sheath is necessary for pyocin to manifest its killing activity toward bacteria.

The conclusions drawn above for R-type pyocins are comparable with those done for the infection of *Escherichia coli* with T-even coliphages. Simon and Anderson (22) demonstrated that the infection of *E. coli* with T2 and T4 bacteriophages was initiated by the attachment of the phages' long tail fibers to the bacterial cells. Attachment of isolated T4 tail fibers to a lipoplysaccharide fraction prepared from sensitive bacteria was reported by Wilson et al. (24). T4 bacteriophages lacking gene 11 and gene 12 products can attach to the bacterial surface by their long tail fibers but they have a severely reduced probability of contracting their sheath, and, as a consequence, they do not inject phage deoxyribonucleic acid into the bacteria or kill cells, as reported by Simon et al. (23).

Relationship among R-type pyocins. All R-type pyocins are indistinguishable on the morphological viewpoint (8), and genes determining the production of pyocin R1, R2, and R3 are mapped at the same position on the chromosome of *P. aeruginosa* (10–13). Detailed studies

on the serological properties and the subunit composition of these pyocins, however, revealed fine differences among them (manuscript in preparation). Pyocin R2 differs from pyocin R1 in some serum blocking antigen(s), which are located at the distal tip of the fibers. Subunits composing pyocin R2 are the same with pyocin R1 except a minor subunit, no. 14.

In this paper, we have added evidence that supports the close relationship between different groups of R-type pyocins. P15-16 particles, which are originated from pyocin R1, could be converted to active pyocin by incubation with the extracts prepared from certain pyocin R2-less mutants. The results indicate the exchangeability of parts between pyocin R1 and R2.

Morphogenesis of R-type pyocins. In vitro complementation assays were introduced first by Edgar and Wood (3) to study the morphogenesis of T4 bacteriophage. A similar technique was used to obtain information on the morphogenesis of R-type pyocins. We succeeded at in vitro complementation between P15-16 and certain mutants, such as M188, but the complementation with other combinations of the pyocin mutants was unsuccessful.

All the experimental results support the model that defective particles produced by P15-16 are converted to active pyocin particles by assemblage of missing sheath proteins. Efficiency of the reaction in the presence of excess sheath proteins was roughly estimated as follows. The concentration of P15-16 particles in P15-16 lysate was estimated to be approximately one-tenth of that of pyocin particles in P15 lysate (Table 2 and Fig. 2a). Pyocin activities appeared as a consequence of the complementation reaction were approximately 4×10^3 U per ml of added P15-16 lysate (see Fig. 9) and corresponded to about one-tenth of pyocin activity found in P15 lysate (usually 2×10^4 to 8×10^4 U/ml). Therefore, the efficiency of the reaction was very high.

Polysheath, which is produced by M188 or by other mutants (Table 2), must be an aberrant structure probably formed by self-assembly of sheath proteins in the absence of a proper core structure and has a high tendency to form a random aggregate. It seems unlikely that polysheath itself is a precursor which reacts with a P15-16 particle. Free sheath proteins not yet assembled to polysheath would assemble along the core tubes of P15-16 particles.

It is not clear if P15-16 particle is a real intermediate in the normal pathway of pyocin morphogenesis. A particle with a sedimentation coefficient of about 40S has been found during the course of pyocin R1 production in P15 cell (18). The particle is precipitable by anti-pyocin R1 serum but not by anti-sheath serum. These results suggest the existence of an intermediate close to P15-16 particle in the course of pyocin R1 morphogenesis in vivo, but the subunit proteins composing the 40S particle found in vivo have not been determined.

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