Studies on the Pyocins of *Pseudomonas aeruginosa*: Production of Contractile and Flexuous Pyocins in *Pseudomonas aeruginosa*

By J. R. W. GOVAN

*Department of Bacteriology, Medical School,
University of Edinburgh, Edinburgh*

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SUMMARY

Strains of *Pseudomonas aeruginosa* (*P. pyocyanea*) were examined for pyocin production by means of a standard pyocin-typing technique and also a modified method which depends on the ability of some pyocins to pass through a cellulose acetate membrane. The results suggested that strains could produce (1) pyocins incapable of spontaneous passage through the membrane, (2) pyocins capable of passage through the membrane, (3) both kinds of pyocin simultaneously. Electron microscopy revealed that pyocins in the first circumstance included contractile pyocins and those in the second included flexuous, rod-like particles. *P. aeruginosa* 430 produced both contractile and flexuous pyocins simultaneously. The pyocins could be separated by absorption with sensitive bacteria or by gel filtration. The two pyocins differed serologically and in their range of inhibitory activity against strains of *P. aeruginosa*.

INTRODUCTION

In studies of *Pseudomonas aeruginosa* by means of the standard pyocin-typing technique (Gillies & Govan, 1966) differences in the extent of inhibition zones of the eight indicator strains suggested that some strains were capable of producing simultaneously pyocins of differing diffusibility. These observations led to the development of a method of subdividing type 1 strains into eight subtypes by means of five additional indicator strains (Govan & Gillies, 1969). The use of a cellulose acetate membrane in pyocin typing was suggested to facilitate removal of macroscopic growth of the pyocinogenic strain (Kohn, 1966). However, applying the membrane modification to the standard typing technique has not proved satisfactory because many pyocins appear incapable of spontaneous passage through the membrane (Govan, 1968; MacPherson & Gillies, 1969).

In addition to contractile pyocins such as R (Ishii, Nishi & Egami, 1965), C9 (Higerd, Baechler & Berk, 1967) and several others (Govan, 1968; 1974), a flexuous, non-contractile type, pyocin 28, has been described (Takeya, Minamishima, Amako & Ohnishi, 1967) and also a non-particulate type designated pyocin S (Ito, Kageyama & Egami, 1970). Amako, Yasunaka & Takeya (1970) observed contractile and flexuous bacteriocins in mitomycin C-induced lysates of *Pseudomonas fluorescens*. Ito *et al.* (1970) reported the production of contractile and S-type pyocins together in *P. aeruginosa* strains M11 and P28 after induction with mitomycin C or ultraviolet irradiation. The simultaneous production of contractile and flexuous pyocins by *P. aeruginosa* 430 is described in this paper.

METHODS

Bacteria. The following pyocinogenic strains of *Pseudomonas aeruginosa* were investigated: 21, 355, 450, 318, 295, 430 and 485. The indicator strains of *P. aeruginosa* used to detect pyocin activity were those of Gillies & Govan (1966), numbered 1 to 8.

Pyocin typing. Pyocin typing was performed as previously described (Gillies & Govan, 1966).

Pyocin typing with cellulose acetate strips. Pyocin typing was also carried out with cellulose acetate electrophoresis strips (Oxoid, pore size approx. $0.45\ \mu\text{m}$) and a modification of the method of Kohn (1966). A strip of cellulose acetate was placed on the surface of a tryptone soya agar plate incorporating 7 % (v/v) defibrinated horse blood and the pyocinogenic strain of *Pseudomonas aeruginosa* was inoculated directly on to the acetate surface with a sterile swab. After 18 h incubation at $32\ ^\circ\text{C}$ the strip and macroscopic growth were removed and the surface of the medium exposed to chloroform vapour for 15 min. The eight indicator strains of *P. aeruginosa* (4 h nutrient broth cultures at $37\ ^\circ\text{C}$) were then streaked at right-angles over the growth area of the pyocinogenic strain and the plates reincubated overnight at $37\ ^\circ\text{C}$.

Production and purification of pyocins. Extraction, purification and assay of pyocins from induced and non-induced cultures of pyocinogenic strains were carried out as described earlier (Govan, 1974).

Separation of pyocins by absorption. Cells of *Pseudomonas aeruginosa* were grown overnight at $37\ ^\circ\text{C}$ on nutrient agar plates. The growth from four plates was harvested, washed in sterile saline (0.85 %) and centrifuged at $1200\ g$ for 20 min. Ten ml of pyocin preparation were added to the pellet of bacteria and the contents were mixed. The mixture was left at room temperature for 20 min and the bacteria removed by centrifugation. The supernatant was treated with 5 % (v/v) chloroform to kill any remaining viable bacteria and assayed for residual pyocin activity.

Centrifugation of pyocins through a sucrose density gradient. Partially purified induced pyocin preparations from *Pseudomonas aeruginosa* 485 (0.2 ml volumes) were centrifuged for 2 h at $130000\ g$ through a linear sucrose gradient (10 to 40 %) in a refrigerated Spinco SW 50 rotor. Fractions (0.25 ml) of the gradient were assayed for pyocin activity against indicator strains 1 and 5.

Separation of pyocins by gel filtration. Attempts were made to separate induced pyocins from *Pseudomonas aeruginosa* 430 by means of agarose gels (Pharmacia, Uppsala, Sweden). Columns were prepared with either Sepharose 2B (exclusion limit mol. wt 25×10^6) or 4B (exclusion limit 3×10^6). Both gels were provided in liquid phase; 0.02 % sodium azide was incorporated. The gels were washed several times in 0.01 M-tris (hydroxymethyl) methylamine buffer (pH 7.5) containing 0.01 M- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.01 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, after which the slurry was used to prepare a column ($35 \times 2.5\ \text{cm}$) incorporating a sample applicator. After 300 ml of buffer had been passed through the column, 3 ml of partially purified, induced but unseparated pyocin 430 (400000 units/ml) were pipetted on to the surface of the sample applicator. After allowing adsorption for 20 min, the column was eluted with the same buffer at a pressure head of 7 cm. Fractions were collected in 5 ml volumes and assayed for pyocin activity.

Treatment of pyocin with antiserum or lipopolysaccharide. Antiserum to pyocin was prepared and the actions of antiserum and lipopolysaccharide (LPS) on pyocin activity were investigated as described in the previous paper (Govan, 1974).

Immunodiffusion. Immunodiffusion experiments were carried out by using a modification

Table 1. *Inhibition patterns produced by pyocinogenic strains of Pseudomonas aeruginosa against indicator strains*

Pyocinogenic strain	Pyocin-typing technique	Indicator strains							
		1	2	3	4	5	6	7	8
21	S	+	+	+	+	+	-	+	+
	M	-	-	-	-	-	-	-	-
355	S	+	+	+	-	+	-	+	-
	M	-	-	-	-	-	-	-	-
450	S	+	-	+	+	-	-	+	+
	M	+	-	+	+	-	-	+	+
318	S	-	-	-	-	+	-	-	-
	M	-	-	-	-	+	-	-	-
295	S	-	-	+	-	-	-	+	-
	M	-	-	+	-	-	-	+	-
430	S	+	+	+	+	+	+	+	+
	M	-	-	-	-	-	+	-	-
485	S	+	+	+	-	+	-	+	-
	M	-	-	-	-	+	-	-	-

S, Standard typing technique (Gillies & Govan, 1966).

M, Modified technique using cellulose acetate (see text for details).

+, Inhibition of indicator strain; -, no inhibition.

of the method of Ouchterlony (1949). A solution of 2 % Oxoid Ionagar no. 2 in 0.9 % saline and containing 0.2 % sodium azide was steamed for 1 h. A mixture of 9 ml of 0.1 M-citric acid and 41 ml of 0.2 M- Na_2HPO_4 was heated to 90 °C and mixed with 50 ml of agar solution. The molten agar was then dispensed in 10 ml amounts in plastic Petri dishes 8.5 cm in diameter. After the agar had set, a central well and six equidistant lateral wells were cut from the agar with a gel cutter (Shandon, London); the central well was filled with antiserum and various antigens were distributed in the lateral wells. The plates were placed in a moist container at 4 °C and photographed after seven days.

Electron microscopy. Electron microscopy was carried out by using techniques described earlier (Govan, 1974).

RESULTS

Pyocin typing by the standard method and the modified cellulose acetate technique

The results of typing *Pseudomonas aeruginosa* strains 21, 355, 450, 318, 295, 430 and 485 by the standard technique (Gillies & Govan, 1966) and a modified technique using cellulose acetate (Kohn, 1966; Govan, 1968) are shown in Table 1.

By using the standard pyocin-typing technique the patterns of inhibition against the eight indicator strains of *Pseudomonas aeruginosa* showed that the strains belonged to pyocin types 1, 3, 16, 5, 17, 10 and 3 respectively. When using the cellulose acetate technique, pyocins of strains 21 and 355 no longer inhibited any indicator strain whilst strains 450, 318 and 295 showed no change in the patterns of inhibition. Strain 430 inhibited all eight indicator strains in the standard typing technique (Fig. 1) whereas only indicator strain 6 was inhibited in the modified method (Fig. 2). Strain 485 inhibited strains 1, 2, 3, 5 and 7 by the standard method but only indicator strain 5 when cellulose acetate was used.

Strains 21, 355 and 430 produced the contractile pyocins described previously (Govan, 1974). Non-induced cultures of strains 450, 318 and 295 produced low titres of pyocin

Fig. 1. Pyocin-typing plate of *Pseudomonas aeruginosa* strain 430 by the standard pyocin-typing technique, showing inhibition of all eight indicator strains.

Fig. 2. Pyocin-typing plate of *Pseudomonas aeruginosa* strain 430 with the cellulose acetate modification; only indicator strain 6 is inhibited with this method.

Table 2. *Inhibitory activity of induced unseparated pyocins 430 and 485 after absorption with bacteria of Pseudomonas aeruginosa*

Pyocin	Absorbed with bacteria of indicator strain	Pyocin activity (units/ml) against indicator strain			
		1	5	6	8
430	6	100 000	100 000	0	100 000
430	8	0	0	100 000	0
430	C	100 000	100 000	100 000	100 000
485	1	0	1600	0	0
485	5	0	0	0	0
485	C	100 000	100 000	0	0

C, control: no absorption carried out.

Fig. 3. Assay of induced pyocin preparations from *Pseudomonas aeruginosa* strain 485 after centrifugation through a sucrose density gradient. Fractions were assayed for pyocin activity against indicator strain 1 (a) and indicator strain 5 (b): ○—○ unseparated pyocin 485; ●—● pyocin 485 after absorption with bacteria of indicator strain 1.

activity (approximately 3200 units/ml) after growth in liquid or solid media, but after induction with mitomycin C crude lysates of each strain had titres of 100 000 to 400 000 units/ml. Electron microscopy of partially purified lysates revealed only a single type of particle; this was a flexuous rod approximately 10 nm wide and 100 to 200 nm long. No change in morphology of the pyocin particles was observed after their attachment to the surface of a sensitive bacterium.

Absorption of pyocin preparations derived from strains 430 and 485 by the eight indicator strains

Unabsorbed pyocin 430 inhibited all eight indicator strains, but after absorption with washed cells of indicator strain 6 pyocin activity remained towards the seven other strains, i.e. 1, 2, 3, 4, 5, 7 and 8. After absorption with each of these strains activity towards indicator strain 6 remained.

Unabsorbed pyocin 485 inhibited indicator strains 1, 2, 3, 5 and 7 but did not inhibit strains 4, 6 and 8 and absorption of this pyocin with strains 4, 6 or 8 produced no alteration

Fig. 4. *Pseudomonas aeruginosa* indicator strain 6 after treatment with induced flexuous pyocin 430f. The bacterial surface is surrounded with flexuous, rod-like pyocin particles. Bar marker = 200 nm.

in pyocin activity. Absorption with indicator strains 1, 2, 3 or 7 did not remove pyocin activity towards indicator strain 5 but absorption with indicator strain 5 removed pyocin activity to all the indicator strains.

Partially purified induced pyocin preparations (100000 units/ml) were obtained from strains 430 and 485 and a quantitative assay of pyocin activity was carried out after absorption with washed bacteria of indicator strains 6 and 8 or 1 and 5 respectively (Table 2).

Fig. 5. *Pseudomonas aeruginosa* indicator strain 8 after treatment with induced contractile pyocin 430c. Many contracted pyocin particles can be seen adsorbed to the bacterial surface and in the vicinity of the bacterium. Bar marker = 200 nm.

Absorption failed to separate discrete pyocins from pyocin 485. Absorption with indicator strain 1 resulted in much reduced pyocin activity towards indicator strain 5; absorption with bacteria of the latter removed all pyocin activity. An attempt was therefore made to separate the pyocins of strain 485 by centrifugation through a sucrose density gradient. Unseparated induced pyocin 485 and a preparation obtained after absorption with indicator strain 1 were centrifuged. A fraction which inhibited indicator strain 1 was found only at the 30 % sucrose level, whereas pyocin activity against indicator strain 5 was found at both the 10 and 30 % sucrose levels (Fig. 3). Absorption experiments with pyocin 430 showed that a pyocin with specific activity against indicator strain 6 could be obtained after absorption with bacteria of strain 8; absorption with strain 6 left undiminished pyocin activity towards strains 1, 5 and 8. Both pyocins of strain 430 were inducible with mitomycin C and were examined in greater detail.

Ion-exchange chromatography

When unseparated induced pyocin 430 was chromatographed on DEAE-cellulose (Govan, 1974) pyocin activity against indicator strains 6 and 8 was eluted as a single peak at 0.2 to 0.3 M-sodium chloride.

Gel filtration

Pyocin 430 active against indicator strain 6 but not strain 8, was eluted from a column of Sepharose 2B in fraction 16 (80 ml) and reached a maximum (50000 units/ml) at fraction 20 (100 ml). Even after elution with 250 ml buffer, no activity against strain 8 was noted. When the eluant flow through the column was reversed, pyocin activity was detected commencing in fraction 6. This activity was directed against strain 8 only and reached a maximum of 3200 units/ml. Attempts to separate the pyocins of strain 430 by using Sepharose 4B were unsuccessful.

Electron microscopy

After gel filtration of unseparated, induced pyocin 430, electron microscopy of fractions active towards indicator strain 6 revealed only flexuous pyocin particles morphologically similar to those of strains 450, 318 and 295; this flexuous pyocin from strain 430 will be referred to as pyocin 430f. The fractions containing pyocin activity directed against strain 8 showed only contractile pyocin particles; designated pyocin 430c. Attachment of pyocin 430f to bacteria of strain 6 (Fig. 4) and pyocin 430c to bacteria of strain 8 (Fig. 5) was noted. No attachment of pyocin 430f to bacteria of strain 8 or pyocin 430c to bacteria of strain 6 was observed.

Electron microscopy of unseparated induced pyocin 485 revealed contractile pyocins and occasionally flexuous rods; these were not investigated further.

Serological investigations of pyocins 430f and 430c

Neutralization of pyocin activity. Antiserum prepared against unseparated induced and purified pyocin 430 was examined for neutralizing antibodies to pyocins 430f and 430c by using indicator strains 6 and 8 respectively. A neutralization titre of 2000 was found against both pyocins. No neutralization of pyocin activity was observed when using pre-immune sera.

Gel diffusion. By means of a double diffusion technique, antiserum against unseparated induced and purified pyocin 430 was examined against pyocins 430f and 430c. Pyocin 430f gave a single precipitin line whereas 430c produced three distinct lines. When unseparated pyocin 430, pyocin 430f and pyocin 430c were placed in adjacent wells (Fig. 6) the single

Fig. 6. Antigen-antibody precipitation reaction of induced pyocins in agar gel. The central well contains antiserum to unseparated pyocin 430, and (a) unseparated pyocin 430, (b) flexuous pyocin 430f, (c) contractile pyocin 430c, and (d) unseparated pyocin 485.

Table 3. *Inhibitory activity of induced pyocins from Pseudomonas aeruginosa 430 after treatment with LPS from indicator strains 6 and 8*

Pyocin	Treated with LPS from indicator strain	Pyocin activity (units/ml) against indicator strain	
		6	8
430*	6	50000	50000
430*	8	50000	0
430*	C	50000	50000
430f	6	50000	0
430f	8	50000	0
430f	C	50000	0
430c	6	0	50000
430c	8	0	0
430c	C	0	50000

* Unseparated pyocin 430.

C, control: pyocin preparation treated with portion of 0.1 M-ammonium acetate.

Fig. 7. Induced pyocin 430f adsorbed to LPS from *Pseudomonas aeruginosa* indicator strain 6. Bar marker = 200 nm.

line of 430f showed identity with one of the four lines produced against unseparated pyocin 430; similarly one of the three lines produced against pyocin 430c showed identity with another of the lines obtained with unseparated pyocin 430. The precipitin line of pyocin 430f showed a reaction of non-identity with those of pyocin 430c.

The effect of LPS from indicator strains 6 and 8 on the activity of pyocins 430f and 430c

Unseparated induced pyocin 430, pyocin 430f and pyocin 430c were treated with LPS from indicator strains 6 and 8 and assayed for pyocin activity (Table 3).

Pyocin 430c, when present in unseparated induced pyocin 430 preparations, or as a separated fraction, was completely inactivated by LPS from indicator strain 8; no inactiva-

Fig. 8. Rounded fragment of LPS from *Pseudomonas aeruginosa* indicator strain 6 in the presence of unseparated induced pyocin 430 containing both flexuous (f) and contractile (c) pyocins. The selective adsorption of flexuous pyocin particles to the LPS is visible. Bar marker = 200 nm.

Fig. 9. Contracted particles of induced pyocin 2285 adsorbed to LPS from *Pseudomonas aeruginosa* indicator strain 6. Bar marker = 200 nm.

tion of pyocin 430c was noted after treatment with LPS from strain 6. In contrast, pyocin 430f which specifically inhibited strain 6 was unaffected by LPS extracts from strain 6 or 8; the biological activity of the LPS fraction from strain 6 was confirmed by its ability to inactivate the lethal activity of a contractile pyocin from *Pseudomonas aeruginosa* strain 2285.

Electron microscopy of pyocin – LPS interaction. When unseparated induced pyocin 430 or induced pyocin 430c was treated with LPS extracted from strain 8 and examined in the electron microscope, contracted pyocin particles were found attached to the surface of the

LPS fragments. Although induced pyocin 430f did not appear to be inactivated by treatment with LPS from strain 6 the flexuous particles were found attached to LPS fragments (Fig. 7), and indeed when unseparated induced pyocin 430 was treated with LPS from strain 6, selective attachment of the flexuous 430f pyocin particles appeared to take place (Fig. 8). The biological activity of LPS from indicator strain 6 was confirmed in the electron microscope by its ability to absorb a contractile pyocin 2285 (Fig. 9). Pyocin 430c or 430f particles did not absorb to LPS from strains 6 or 8 respectively.

DISCUSSION

These results suggest that a modified typing technique employing a cellulose acetate membrane might prove a useful preliminary step in screening pyocinogenic strains of *Pseudomonas aeruginosa* for the production of contractile or flexuous pyocins.

Strains 21 and 355 show pyocin activity when examined by the standard typing technique but not when examined by the modified typing technique. Electron micrographs have shown (Govan, 1968; 1974) that these strains produce contractile pyocins. This type of particle may absorb to or be inactivated by the cellulose acetate membrane, because the dimensions of the particles (100×15 nm) should otherwise have allowed their passage through a membrane with a pore size of approximately $0.45 \mu\text{m}$. Flexuous pyocins were produced by pyocinogenic strains 450, 318 and 295 and the inhibition pattern produced by these strains was unaffected by cellulose acetate. Recent investigations indicate that, in addition to flexuous pyocins, non-particulate S-type pyocins can also pass spontaneously through these cellulose acetate membranes (J. R. W. Govan, unpublished).

The use of the modified technique suggested that strains 430 and 485 produce more than one type of pyocin simultaneously and this possibility was examined.

Pseudomonas aeruginosa 430 produced both contractile and flexuous pyocins whose production could be increased by induction with mitomycin C. Since the inhibitory activity of these pyocins was directed towards different indicator strains the pyocins could be separated by absorption with sensitive bacteria. Gel filtration with Sepharose 2B, however, provided a method for the preparation of purified flexuous pyocin. *P. aeruginosa* 485 produced an inducible contractile pyocin active against strains 1, 2, 3, 5 and 7 and also a non-inducible pyocin which inhibited only indicator strain 5. The non-inducible pyocin could be isolated from unseparated pyocin 485 by removing the contractile pyocin by absorption with bacteria of indicator strains 1, 2, 3 or 7. Indicator strain 5 could not be used since it possessed receptors for both types of pyocin. Gel filtration was not attempted because of the small amount of non-inducible pyocin present, but the two pyocins could be separated by centrifugation through a sucrose density gradient.

The many different patterns of inhibition observed with the pyocin-typing techniques must result from production of different contractile, flexuous and non-particulate pyocins or from combinations of these. A study of these different pyocins may allow interpretation of the inhibition patterns in terms of production of individual pyocins. Subdivision of strains of *Pseudomonas aeruginosa* belonging to pyocin type 1 has already been reported (Govan & Gillies, 1969). Current investigations (J. R. W. Govan, unpublished) suggest that many other pyocin types of *P. aeruginosa* are capable of subdivision when the individual pyocins responsible are examined more closely.

It seemed worthwhile to investigate the possibility that flexuous pyocin particles are precursors of the more complex contractile pyocins especially when both pyocins are produced by the same strain of *Pseudomonas aeruginosa* as in the case of strain 430.

Morphologically, flexuous pyocin particles can often be distinguished from the tail cores of contractile pyocins by their variable length and less rigid appearance. Further evidence that these are two quite different entities is suggested by the following observations. (i) The two pyocins have different spectra of activity and in strain 430 there was not even a sharing of inhibitory activity against any one indicator strain. (ii) The flexuous pyocin could adhere to and kill sensitive bacteria whereas the isolated tail core of the contractile pyocin lost the ability to adsorb to such bacteria (Govan, 1974). (iii) No serological relationship could be demonstrated between the two pyocins of strain 430 by using the gel diffusion technique.

The determination of a chromosomal locus for control of synthesis of the contractile pyocin R2 (Kageyama, 1970*a, b*) suggests that contractile pyocins are the result of prophage alteration. A similar location for factors determining flexuous pyocins would suggest that they originate from prophage elements controlling synthesis of non-contractile bacteriophages of group B (Bradley, 1967). Such bacteriophages are known to exist in *Pseudomonas* (Slayter, Holloway & Hall, 1964; Bradley, 1966) but normally possess a knob-like tip to the tail extremity which is absent in flexuous pyocins.

LPS extracted from sensitive bacteria of *Pseudomonas aeruginosa* neutralizes the inhibitory activity of contractile pyocins by absorbing pyocin particles (Govan, 1974). LPS from indicator strain 6 absorbed and inactivated a contractile pyocin 2285 and had no effect on the inhibitory activity of the flexuous pyocin 430f; this suggests that there was a different chemical receptor for this flexuous pyocin. However, electron micrographs show the apparent attachment of pyocin 430f to LPS fragments from indicator strain 6. Since the contractile pyocin 430c did not inhibit this strain and did not attach to intact bacteria or LPS fragments from indicator strain 6, the adsorbed particles could not be the sheathless remains of pyocin 430c. Perhaps the bacterial receptors for the flexuous pyocin contain LPS but attachment is reversible without concomitant loss of inhibitory activity. Further investigations are required to determine the nature of these bacterial receptors.

A continued study of the incidence, nature and mode of action of pyocins is important in studies of the epidemiology, genetics and physiology of *Pseudomonas aeruginosa*.

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