Receptor Sites for R-type Pyocins and Bacteriophage E79 in the Core Part of the Lipopolysaccharide of *Pseudomonas aeruginosa* PAC1

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INTRODUCTION

The bacteriocins produced by *Pseudomonas aeruginosa* are known as pyocins and fall into two classes. The S-type are characterized by their sensitivity to proteolytic enzymes and their lack of fine structure. The R-type pyocins are resistant to these enzymes and have structures similar to bacteriophage tails (for review, see Holloway & Krishnapillai, 1975).

Ito & Kageyama (1970) and Kageyama (1975) have divided the R-type pyocins into five groups (R1 to R5) depending on their activity against different strains of *P. aeruginosa* p14. They suggested that sensitivity might depend on the presence of a specific receptor in the wall and proposed a scheme whereby the receptors for the R3, R2, R4 and R5 pyocins were arranged linearly in a projection from the bacterial surface; the R1 site would project as a branch between the R2 and R5 sites. Material with pyocin receptor activity has been isolated and shown to consist mainly, if not entirely, of lipopolysaccharide (LPS) (Ikeda & Egami, 1969; Govan, 1974), but the part of the LPS responsible has not been identified.

Recently Jarrell & Kropinski (1977) have shown that the bacteriophage E79 was almost completely neutralized by crude LPS preparations from *P. aeruginosa* PAO, but not by LPS from a bacteriophage-resistant mutant derived from it. They suggested that the bacteriophage E79 receptor was LPS but did not define the part responsible.

The work described here was undertaken to see whether Kageyama's classification of R-type pyocins applied to strain PAC1 and its LPS-defective mutants and to try to define the components responsible for sensitivity to R-type pyocin and bacteriophage E79. A preliminary account of some of this work has been published (Meadow & Wells, 1977).

METHODS

Pseudomonas aeruginosa strains and growth conditions. The parent strains PAC1 (NCIB 10848) and PAC1R and the LPS-defective mutants PAC556, PAC605 and PAC610 have all been described previously (Koval & Meadow, 1977; Meadow *et al.*, 1978). PAC1R was used as the parent strain for subsequent isolations because it is insensitive to several bacteriophages active against PAC1, but its LPS composition is indistinguishable from that of the latter (Chester & Meadow, 1975). PAC611 and PAC609 were isolated from PAC1R by selection for resistance to the pyocin produced by *P. aeruginosa* NIHD.

Pyocin-producing strains were obtained as follows: strains ISE, sero6, R21, PP430, IS4, ISD, F31, ISC and ISA from Dr J. Govan, Medical School, Edinburgh; strains QED, NIH26, NIHD, QEE and NIH18 from Dr A. Ramping, Queen Elizabeth Hospital, Birmingham; strains M12, P15, P28 and SLM6 from Dr M. Kageyama, Mitsubishi-Kasei Institute, Tokyo, Japan; strain PAT1 from Professor B. W. Holloway, Monash University, Melbourne, Australia; and strain P16 from Dr N. Suzuki, Institute of Medical Sciences, University of Tokyo, Japan.

The strains were grown in nutrient broth or on nutrient agar at 37 °C and their serotypes were determined as previously described (Koval & Meadow, 1977).

Preparation of R-type pyocins. Crude pyocin preparations were made by adding mitomycin C (1 µg per ml culture) to producer strains growing exponentially (A_{670} 0.3) in nutrient broth with shaking at 37 °C. After

4 h incubation, the bacteria were deposited by centrifugation (4000 g, 10 min) and the residual bacteria in the supernatant were killed by adding chloroform (5 %, v/v). Sterility of the lysate was confirmed by streaking a loopful on to a nutrient agar plate and incubating it overnight at 37 °C. These mitomycin lysates contained both S- and R-type pyocins and often bacteriophages as well. The S-type pyocins were destroyed by trypsin treatment (100 µg ml⁻¹, 3 h at 37 °C). None of the preparations contained bacteriophages active against PAC1R, although bacteriophages were detected using other indicator strains and were seen in electron microphotographs. The pyocins appeared similar to those shown by Govan (1974). The trypsin-treated mitomycin C lysates, designated pyocin preparations, were titrated with PAC1R as indicator using doubling dilutions essentially as described by Govan (1974).

Isolation of pyocin- and bacteriophage-resistant mutants. The methods used were those of Koval & Meadow (1975) except that exponential phase cultures (A_{670} 1.0) were used both for mutant isolation and binding studies. The mutants were classified as resistant, rather than tolerant (Holloway & Krishnapillai, 1975), if they did not bind the pyocin and hence change the titre. The effectiveness of this technique was confirmed in a few cases by electron microscopy which showed that sensitive and tolerant strains bound pyocins while resistant strains did not.

Electron microscopy. Bacteria and pyocin preparations were viewed after negative-staining as described previously (Meadow *et al.*, 1978).

Isolation and analysis of LPS. This was purified from phenol extracts of isolated walls and, after acid hydrolysis, fractionated by elution from Sephadex G75 or Biogel P6 (Koval & Meadow, 1977). The methods for estimating glucose, rhamnose, heptose and amino compounds in each fraction were all as described previously (Koval & Meadow, 1977).

RESULTS

Sensitivity of PAC1 strains to R-type pyocins

The parent strains PAC1 and PAC1R were both sensitive to pyocins from each of the five R-types defined by Kageyama (1975), but their LPS-defective mutants PAC605, PAC556 and PAC610 were resistant to some or all of the pyocin types. These mutants all lacked the O-antigenic side-chain of the LPS, but differed in the amount of residual core structure suggesting that the pyocin receptor sites might involve the core part of the LPS. These mutants did not allow us to distinguish between all pyocin types and so mutants resistant to the R2-type pyocin from strain NIH8 were isolated. Most of those selected were resistant to all pyocin types but two, PAC609 and PAC611, retained their sensitivity to the R5 pyocin and enabled us to distinguish four of the five types (Table 1). Attempts to isolate mutants of PAC1 and PAC1R which were sensitive to R2 pyocins but resistant to the R4-type were not successful. Only one strain thought to produce an R4 pyocin was available and subsequent work was limited to the other four types.

Nineteen strains of *P. aeruginosa* producing pyocins active against *P. aeruginosa* PAC1 were obtained from other laboratories. The R-type pyocins produced were tested against the strains shown in Table 1. All could be assigned to one of the R-types defined by Kageyama for strain P14 and our results with the PAC1 strains confirmed his classification in 13 out of the 14 preparations previously classified. The exception was the pyocin from strain NIH18 classified as R5 (Kageyama, 1975). In our hands this behaved like the R2 pyocin from M12, mutants PAC609, PAC611 and PAC605 all being resistant to it. Kageyama (personal communication) has now re-tested this strain and shown it to belong to the R2 class. Of the previously unclassified pyocins, those from QEE and sero6 behaved as R2 pyocins, while QED, F31 and P16 produced R3, R1 and R5-type pyocins, respectively.

Our results with PAC1 and its pyocin-resistant mutants are compatible with the sequence of receptor sites proposed for strain P14.

Sensitivity to bacteriophage E79

Mutants of PAC1R resistant to bacteriophage E79 were readily isolated and like the LPS-defective mutants appeared smooth on plates. Twenty E79-resistant mutants were isolated and tested for their sensitivity to the various pyocin types. The majority were resistant to all pyocin types, but two remained sensitive to the R5 pyocins thus distinguishing between

Table 1. *Relationship between sensitivity to R-type pyocin and to bacteriophage E79 and lipopolysaccharide structure in mutants of P. aeruginosa PAC1*

	PAC strain					
	1 and 1R	610	609	556	611	605
Serotype	3	NT	3	NT	NT	NT
EM appearance	Smooth	Rough	Smooth	Rough	Rough	Rough
Response to R-type pyocins*						
R3 (ISE)	S	R	R	S	R	R
R4 (P28)	S	S	R	S	R	R
R2 (M12)	S	S	R	S	R	R
R1 (P15)	S	S	S	R	R	R
R5 (SLM6)	S	S	S	S	S	R
Response to bacteriophage E79	S	S	S	S	R	R
Molar ratios of components of core fraction of LPS†						
Glucose	5.5	4.5	3.5	4.7	1.1	0
Rhamnose	2.6	1.8	2.5	0	0	0
Galactosamine	1.0	1.0	1.0	1.0	1.0	1.0
Alanine	1.8	1.3	1.9	1.2	1.4	1.6
Heptose	2.1	2.4	2.8	1.7	1.8	1.7

NT, Not typable; S, sensitive; R, resistant.

* Producer strains are indicated in parentheses.

† The core fraction was the L2 fraction recovered from the partially degraded polysaccharide after elution from Biogel P6. Molar ratios are expressed relative to galactosamine (= 1.0).

the E79 and the R5 pyocin receptors. This distinction was confirmed by the response of the pyocin-resistant mutants to bacteriophage E79 (Table 1).

Purified preparations of LPS at concentrations up to 100 µg ml⁻¹ had no effect on the titre of bacteriophage E79 when tested under the conditions used by Jarrell & Kropinski (1977). Under the same conditions, pyocin preparations were completely neutralized by LPS at 0.5 µg ml⁻¹.

Analysis of the isolated lipopolysaccharides

Mutant PAC609 differed from the other pyocin-resistant mutants in reacting with Habs type 3 antisera suggesting it contained O-polysaccharide side-chains. This was confirmed by separation and analysis of its partially degraded polysaccharide fractions. Separation on Sephadex G75 yielded high molecular weight fractions containing the amino sugars characteristic of the Habs type 3 strains (aminogalacturonic acid and bacillosamine) and indistinguishable both in amount and composition from the same fractions derived from the parent strain. The only apparent difference between PAC609 LPS and that of the parent strains was in the reduced glucose content of the core fraction (Table 1).

PAC611 was untypable by the Habs antisera and separation and analysis of its partially degraded LPS on Sephadex G75 and Biogel P6 confirmed that it contained less than 1% of the O-antigenic side-chains found in the parent strain. The partially degraded polysaccharide from this mutant contained only one component detected by the phenol-sulphuric acid reagent after elution from Biogel P6. Its position corresponded to that of the core fraction (L2) obtained from the parent strains. The PAC611 L2 fraction contained galactosamine, alanine and heptose, but lacked rhamnose and contained less glucose than the L2 fraction from the parent strain. It appeared to differ from the L2 fraction of the most defective mutant PAC605 by a single glucose unit (Table 1).

The LPS of the other resistant mutants has been described previously (PAC610, Meadow *et al.*, 1978; PAC605 and PAC556, Koval & Meadow, 1977); the relevant data are included in Table 1 for comparison.

Fig. 1. Tentative partial structure of the core part of the LPS of *P. aeruginosa* PAC1. Receptor sites for the R-type pyocins and bacteriophage E79 are represented by blocks. Dotted lines show possible lesions of the defective mutants.

DISCUSSION

Our results suggest that the receptor sites for the R-type pyocins and for bacteriophage E79 all involve part of the LPS core, since all resistant mutants lacked one or more core components. Whether the LPS core provides the entire receptor for E79 remains to be seen. Jarrell & Kropinski (1977) neutralized 50% of the activity of bacteriophage E79 with crude LPS [$0.4 \mu\text{g ml}^{-1}$ containing RNA (9.5%, w/w) and protein (2.5%, w/w)] from strain PAO. Using similar conditions and purified LPS from PAC (up to $100 \mu\text{g ml}^{-1}$) we were unable to affect the bacteriophage titre whereas the activity of pyocin preparations was readily neutralized by PAC LPS. Binding of the bacteriophage by LPS may occur only when the residues involved are in a specific conformation which was lost in our purification procedures. Alternatively additional outer membrane components may also be required.

The structure of the polysaccharide portion of the LPS of *P. aeruginosa* has not yet been established but a tentative partial structure has been proposed for a few strains (see Wilkinson, 1978). Our results for PAC1 are compatible with the structure proposed for strain 8505 and enable us to represent each mutant by a single defect relating pyocin and bacteriophage response to LPS core structure (Fig. 1). The only mutant whose defect has so far been defined is PAC556 which cannot synthesize TDPrhamnose and whose LPS has a vacant acceptor site for rhamnose (Asonganyi & Meadow, 1978). The behaviour of this mutant shows the necessity for rhamnose in R1 sensitivity. PAC605 and PAC611 both of which were spontaneous mutants, may each be defective in one of the glucosyl transferases which are presumably required for core biosynthesis, although nothing is yet known about this process in pseudomonads. Comparison of their fractionated LPS suggests that the innermost glucose residue is required for sensitivity to R5 pyocins. The R2 and R3 sites are less well defined by our work but may involve some or all of the glucose disaccharide branch whose exact position is not known. PAC609 was resistant to both R2 and R3 pyocins and was unusual in having a defective core structure without losing its O-antigenicity. Its phenotype could be explained by a defect in the enzyme responsible for transferring the glucose branch. Mutant PAC610 was isolated as a spontaneous serum-resistant mutant from PAC605. Analysis of its LPS fractions suggested that it lacked the O-antigenic chains and one of the glucose residues of the side branch, leading to R3 resistance. This might result from a defect in that glycosyl transferase, but it is difficult to see why it also lacked the O-chains which in PAC609 could be added to a more defective core structure. The R3 resistance appears common and several spontaneous mutants like PAC610 have been isolated. We have at present no satisfactory biochemical or genetic explanation for their properties which clearly require detailed chemical, genetic and biosynthetic study. Until such studies are completed the core structure and mutant lesions proposed must remain tentative.

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