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Correlation Between Lipopolysaccharide Structure and Permeability Resistance in β-Lactam-Resistant *Pseudomonas aeruginosa*

A. J. GODFREY,* L. HATLELID, AND L. E. BRYAN

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1

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Four β-lactam-resistant permeability mutants of *Pseudomonas aeruginosa* PAO503 were studied. The resistance phenotypes were correlated to changes within the lipopolysaccharide. Two of the mutants, PCC1 and PCC19, were shown to differentiate between β-lactams on the basis of relative hydrophobicity. The more hydrophilic antibiotics were less effective at inhibiting these strains. This phenotype was correlated to the presence of mannose, in measurable quantities, in lipopolysaccharide isolated from these strains. The other two strains, PCC23 and PCC100, differentiated between cephem antibiotics on the basis of electrical charge. The presence of a positive charge markedly increased the relative efficiency of an antibiotic. This correlation did not hold for penam derivatives, with the lower-molecular-weight, dianionic molecules being the most effective. Mutants of this type were changed in the amount of "side chain" sugars or, to a minor extent, in their outer membrane protein profiles.

The outer membrane of *Pseudomonas aeruginosa* has long been considered a barrier against β-lactam antibiotics (5, 23, 24). These compounds are believed to permeate through hydrophilic pores in the membrane (10). Hancock et al. (8) have estimated the exclusion limit of the pseudomonal pores to be between 6,000 and 9,000 daltons, in contrast to the pores in *Escherichia coli* and other gram-negative organisms (19) that exclude molecules larger than 600 daltons. A pore size of 9,000 daltons should allow entry of all known β-lactam antibiotics, which range in size from 350 to ca. 800 daltons. *P. aeruginosa* is, however, resistant to a large number of classical β-lactams (3, 4), and the search continues for antibiotics that have usable activity on this organism (4).

Part of the innate resistance in *P. aeruginosa* lies in the possession of an inducible β-lactamase carried chromosomally by most strains (25). The contribution to innate resistance of the outer membrane has been demonstrated by Zimmermann (31), who used hypersensitive mutants to show an increased β-lactam sensitivity spectrum.

The role played by the outer membrane in acquired resistance to β-lactam antibiotics is at the moment based largely upon supposition (1, 9, 10). In an attempt to increase understanding in this area, we investigated a series of nonsibling β-lactam-resistant mutants of *P. aeruginosa* which could not be explained in terms of changes or lack of affinity (5, 6) in the β-lactam targets.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO503 (*met-9011*) (B. W. Holloway, Monash University, Australia) was used as the parent.

Other strains were derived by mutagenesis (see below) with selection for resistance to β-lactams. The methionine requirement of each mutant was confirmed by growth around a methionine disk on minimal agar.

Mutagenesis. A logarithmic-phase culture of PAO503 was treated with 0.05 ml of ethane methanesulfonate per ml for 1 h at 37°C. The washed, treated culture was plated onto brain heart infusion (BHI) agar (Difco Laboratories) supplemented with ticarcillin (PCC23), carbenicillin (PCC1, PCC19), or piperacillin (PCC100) at 50 μg/ml. After overnight growth, resistant colonies were picked and purified by restreaking onto the selective media and then were screened for their resistance profiles on a series of β-lactam antibiotics.

MICs were determined by disk diffusion of each serially diluted test β-lactam on BHI agar seeded with a fresh inoculum (0.1 ml) of the test mutant (optical density at 600 nm, 0.1). The MIC was taken as that concentration of antibiotic which first inhibited growth around the disk (6). Tube dilution MICs were obtained by inoculating 10^5 bacteria into tubes containing 1 ml of BHIB and serial dilutions of the test β-lactam. Cultures were incubated at 37°C for 18 h before evaluation. The MIC was taken as that antibiotic concentration which first inhibited growth.

β-Lactamase production was tested by adding a drop of the chromatogenic cephalosporin nitrocefin to areas of bacterial growth on a BHI agar plate. No color change in 10 min indicated no detectable β-lactamase activity.

β-Lactam antibiotics used. Carbenicillin (Cb; Ayerst Laboratories), ticarcillin (Beecham Laboratories), piperacillin (Pip; Lederle Laboratories), azlocillin (Delbay Research), cefsulodin (Ceba Geigy Corp.), cefotaxime (Roussel), ceftazidime (Glaxo Laboratories), ceftriaxone (Hoffman-La Roche), and nitrocefin (Oxoid) were obtained from the indicated suppliers.

Evaluation of β-lactam targets. The penicillin-binding proteins were assayed as described previously (5, 6).

Determination of a permeability barrier. Permeability of the outer membrane to β-lactam was determined essentially as described by Zimmermann and Rosselet (32).

The incompatibility group P1 plasmid R68.45 was introduced into each mutant by plate mating (7) with selection for resistance to tetracycline (Sigma Chemical Co.) (100 μg/ml), using PAO25 *arg10 leu10* (R68.45) as the donor. Transconjugants were purified on Vogel-Bonner (26) minimal medium plates supplemented with 1 mM methionine and 100 μg of tetracycline per ml. The presence of R68.45 was confirmed by growth on kanamycin (200 μg/ml).

* Corresponding author.

Cultures of the plasmid-containing mutants were grown to logarithmic phase in BHI broth, collected by centrifugation, washed in 0.5 M sodium phosphate buffer (pH 6.8), and suspended in one-tenth the original volume of phosphate buffer. The cultures were split, and one-half of the cells were ruptured by sonication (two 30-s bursts at maximum power) (Bronson Sonifer) on ice.

Enzyme reaction rates were compared as described previously (17).

Isolation of outer membranes. Outer membranes of PAO503 and the mutants were isolated by the method of Hancock and Nikaido (9), with minor modifications. Cells from 4 liters of culture (BHI broth) were pelleted and washed by centrifugation (5,000 rpm, 20 min). Cells suspended in 20 ml of 0.5 M Tris-hydrochloride buffer (pH 7.3)–0.2 mM dithiothreitol were passaged twice through a French pressure cell (18,000 lb/in^2). The preparation was centrifuged at 3,000 rpm for 10 min. The supernatant was collected and loaded onto a discontinuous sucrose gradient. Centrifugations were performed as described previously (9). The pellets were collected, suspended in 0.5 M phosphate buffer (pH 7.5), and stored frozen (−20°C) until required. Protein concentration was determined by the method of Lowry et al. (15).

Outer membrane proteins were fractionated on the sodium dodecyl sulfate-polyacrylamide gel system of Laemmli and Favre (14) and stained with 0.1% Coomassie brilliant blue (Sigma).

Isolation of LPS. Lipopolysaccharide (LPS) was extracted from logarithmic-phase cultures by the phenol extraction technique of Westphal and Jann (27). The percentage of 3-deoxy-D-mannooctulosonic acid (KDO) was estimated by the method of Karkharis et al. (11), using the ammonium salt of KDO (Sigma) as standard.

Neutral sugars were assayed by the cysteine-sulfuric acid technique, using the modification of Wright and Rebers (29).

Analysis of neutral and amino sugars by amino acid analyzer. Lipid A was removed from LPS samples (2 mg/ml) by heating at 100°C for 90 min in 1% acetic acid (reagent grade, Fisher Scientific). The heated suspension was centrifuged at 2,500 rpm for 10 min, and the pellet was discarded. Then 250 μl of the supernatant was hydrolyzed in 1 N HCl (for determination of neutral sugars) or in 6 N HCl (for amino sugar preparation) for 3 h at 100°C in capped tubes. The hydrolyzed samples were dried at 40°C under a stream of nitrogen. Internal standards (100 nmol of fucose for neutral sugars and 20 nmol of mannosamine for amino sugars) were added, and the suspension was redried.

Neutral sugars were converted to glycamine derivatives by the procedure of Perini and Peters (21).

Residues of amino sugars and glycamine samples were reconstituted in 0.5 ml of 0.2 M sodium citrate buffer (pH 3.25), and 50-μl aliquots were injected into a Beckman 121 amino acid analyzer that had been upgraded to 121 M standards and fitted with a Gibson Spectra/glo fluorometer. The column (0.26 by 23 cm) was packed with Durrum DC-6A resin. Glycamine specimens were run at 50°C and were eluted with 90 mM boric acid (pH 8) for 48 min, followed by 90 mM boric acid containing 100 mM sodium chloride (pH 8) to enhance ribose elution. Sugars were detected by reaction with *O*-phthalaldehyde reagent (22) with a fluorometer setting of R = 20. Amino sugars were processed at 65°C and eluted with 90 mM boric acid (pH 8).

RESULTS

MICs of β-lactam antibiotics. The MICs against the strains described above are shown in Table 1. Resistance was found for β-lactams, other than that used to select the mutant, in each case. The pattern of resistance varied in each mutant. PCC100, for example, exhibited a 30-fold increase in resistance to Pip (the β-lactam on which it was selected) but retained the parental susceptibility to Cb.

The other mutants, PCC1, PCC19, and PCC23, had at least fourfold increases in their levels of resistance by tube dilution to all of the β-lactams tested.

All of the mutants maintained the parental susceptibility to the non-β-lactam antibiotics: tetracycline, tobramycin, streptomycin, gentamicin, neomycin, polymyxin B, chloramphenicol, and rifampin (data not shown).

The mutants and parent had no change in β-lactamase levels when tested with the chromogenic cephalosporin nitrocefin or when ruptured cells were assayed by the iodometric technique, using penicillin G or Pip as the substrate. β-Lactamase could be induced by growth on a suitable inducer in all cases.

Penicillin-binding proteins. The β-lactam targets for the parent and each mutant were assessed by reacting isolated inner membrane preparations with either ^{3}H-labeled penicillin G (New England Nuclear Corp.) or ^{14}C-labeled piperacillin G (New England Nuclear Corp.) or ^{14}C-labeled Pip (a gift

TABLE 1. Resistance levels of PAO503 and the mutant strains to β-lactam antibiotics

Drug	R_m[a]	Electrical charge[b]	Mol wt[c]	MIC against PAO503[d]	Fold increase in MIC against[e]: PCC1	PCC19	PCC23	PCC100
Penams								
Ticarcillin	−0.48	2−	377.45	12.5 (15)	10	8	10	5
Cb	−0.41	2−	375.38	25.0 (31)	20	20	5	0
Azlocilin	0.86	−	460.48	6.2 (3.1)	10	5	40	10
Pip	1.19	−	516.55	1.5 (1.5)	4	4	80	30
Cephems								
Cefsulodin	0.07	2−/+	544.55	1.5 (3.1)	30	30	8	4
Cefotaxime	0.19	−	452.44	1.5 (3.1)	30	30	300	60
Ceftriaxone	0.25	2−	550.54	6.2 (6.2)	8	8	80	80
Cefoperazone	0.29	−	616.61	1.5 (6.2)	8	8	80	80
Ceftazidime	0.5	2−/+	520.53	0.7 (0.7)	8	8	30	8

[a] R_m = log (1/Rf − 1), where R is the distance migrated on a silica gel chromatographic plate in an enclosed chamber, using the method of Biagi et al. (2).
[b] Electrical charge is the probable charge configuration of the β-lactam at neutral pH. −, Negative charge; +, positive charge.
[c] Calculated formular weight of the free acid.
[d] MIC of each β-lactam on disks (micrograms per disk) and in tubes (values in parentheses, micrograms per milliliter) for ~10^5 organisms.
[e] Fold increase in MIC for the mutants over that observed for PAO503.

from Lederle). The fluorographs obtained showed no detectable differences between strains, demonstrating that the target proteins were all present and capable of binding at least two β-lactam antibiotics with affinity equal to that of the parent PAO503 (data not shown). The mutant strains required elevated MICs or Pip (Table 1), showing, since it bound to the same degree in all strains (including the parent), that the resistance did not involve a change in the target proteins.

As further confirmation of this observation, competition studies with Cb, ticarcillin, Pip, and cefsulodin against ^{3}H-labeled penicillin G failed to show any significant differences between the mutants and their parent (data not shown).

Determination of a permeability barrier. Penetration of β-lactam antibiotics was determined by the method outlined by Zimmermann and Rosselet (32), as modified by Nicas and Hancock (17). PAO503 and the mutant strains containing R68.45 were used. The data (Table 2) indicated that all four mutant derivatives had hydrolysis rates for intact cells different from that observed for PAO503 (R68.45). Permeability coefficients (*C*) for Pip, Cb, and penicillin G were calculated by the relationships shown in footnote *b* to Table 2 at a β-lactam concentration of 10 mM. The differences between parent and mutants for the hydrolysis rates (for intact cells) and *C* values observed were thought to reflect different mechanisms of reduced permeability which would presumably be reflected in different susceptibilities to the various β-lactams tested (Table 1). Differences for Pip and penicillin G were significant ($P < 0.001$) by the Student *t* test when the mutants were compared with PAO503. The difference for Cb ($P < 0.10$) was not significant, which may reflect the slow hydrolysis rate for Cb by the TEM-2 enzyme rather than a lack of permeation.

Since decreased permeability was apparently the cause of increased resistance, it remained to determine the mechanism by which this was brought about.

Outer membrane protein profiles. Nicas and Hancock (16) have suggested a role for an outer membrane protein (H1) in resistance to polymyxin B and gentamicin. Similarly, Zimmermann (30, 31) has demonstrated a change in the porin protein associated with increased susceptibility to β-lactams. To test the hypothesis that the permeability barrier in PCC1, PCC19, PCC23, and PCC100 was correlated with a change in outer membrane proteins, outer membrane preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1).

The major bands were identical in each case (Fig. 1). A minor change observed in four independent electrophoresis runs was evident (Fig. 1, arrow) in PCC23. However, changes were not observed in the other strains (PCC19, PCC1, and PCC100).

LPS analysis. We used the method of Wright and Rebers (29) to estimate sugar differences, as described by Kropinski et al. (12), of LPS samples (corrected to contain equivalent concentrations of KDO) from each strain. The shape of each curve was similar.

Differences were not evident when the peaks corresponding to heptoses (505 to 545 nm) (29) were compared, the exception being PCC23, with a heptose peak marginally lower than that observed in the other strains.

Amino acid analysis of LPS. The sugar compositions and amino acid contents of all strains were assayed as their glycamine derivatives (for neutral sugars) or directly in the case of amino compounds. Analyzer results are shown in Table 3. Fucose was included as an internal standard for glycamine derivatives, and mannosamine was used for amino sugars. Neither fucose nor mannosamine was detected in strain profiles run without added standards.

Comparing the molar ratios of sugars considered to be in the core of the LPS molecule with those present only in the side chain saccharides gives an estimation of the degree of "roughness" of the strain in question. Fucosamine, known to be part of the O-antigenic side chain in PAO LPS (13), and rhamnose, part of the core (13, 24), were present in a ratio of 1:1 in PAO503, PCC1, and PCC19, whereas PCC100 had fucosamine present but only in reduced amounts. PCC23 was enriched in side chain material, with a ratio approaching 1:2.

Analysis of core saccharides showed a significant difference in the level of glucose in PCC1. The level of mannose was increased in PCC1 and PCC19, as was galactose in PCC1. Other differences occurring in the core were increased percentages of alanine in PCC19 and PCC100. The molar ratio of alanine compared with rhamnose for PCC19 was approximately 2:1, whereas a 1:1 ratio was observed in PAO503. A reduction in the percentage of phosphate was observed in PCC23, indicating a change within the core region (28).

DISCUSSION

The outer membrane of gram-negative bacteria has been postulated to be a major barrier to antibiotic penetration (4,

TABLE 2. Permeability coefficients and hydrolysis rates for Cb, Pip, and penicillin G

Strain	Cb		Pip		Penicillin G	
	Hydrolysis rate[a]	Permeability coefficient[b]	Hydrolysis rate	Permeability coefficient	Hydrolysis rate	Permeability coefficient
PAO503 (R68.45)	2.18 ± 0.92	1.1	7.05 ± 0.67	7.7	23.93 ± 1.60	18.4
PCC1 (R68.45)	1.09 ± 0.79	0.5	2.45 ± 0.78	2.4	1.97 ± 0.62	1.3
PCC19 (R68.45)	1.12 ± 0.84	0.5	1.75 ± 0.05	1.7	2.88 ± 0.35	1.9
PCC23 (R68.45)	1.17 ± 0.14	0.6	0.62 ± 0.6	0.6	0.41 ± 0.11	0.3
PCC100 (R68.45)	2.05 ± 0.37	1.0	2.25 ± 0.3	2.2	0.67 ± 0.23	0.4

[a] Hydrolysis rate for intact cells, expressed as nanomoles per minute per milligram of cells (dry weight). Means ± standard deviations are shown from a total of at least four independent determinations.

[b] Permeability coefficients = 10^{-4} per second per milligram of cells (dry weight). Permeability coefficients (*C*) are calculated from the following relationships:

$$V_i = C\,(S_o\text{-}S_i) \text{ and } [S_i] = V_i/V_d\left(\frac{K_m \cdot [S_o]}{K_m + [S_o] - V_i/V_d \cdot [S_o]}\right)$$

where V_i is the hydrolysis rate for intact cells, V_d is the hydrolysis rate for disrupted cells, $[S_i]$ is the concentration of the substrate in the periplasm, $[S_o]$ is the external concentration of substrate, and K_m is the Michaelis-Menten constant for the TEM-2 enzyme with the different substrates. Hydrolysis rates for lysed cells preparations were: Pip, 50.75 nmol/min per mg of cells (dry weight); Cb, 15.69 nmol/min per mg: penicillin G, 141.21 nmol/min per mg.

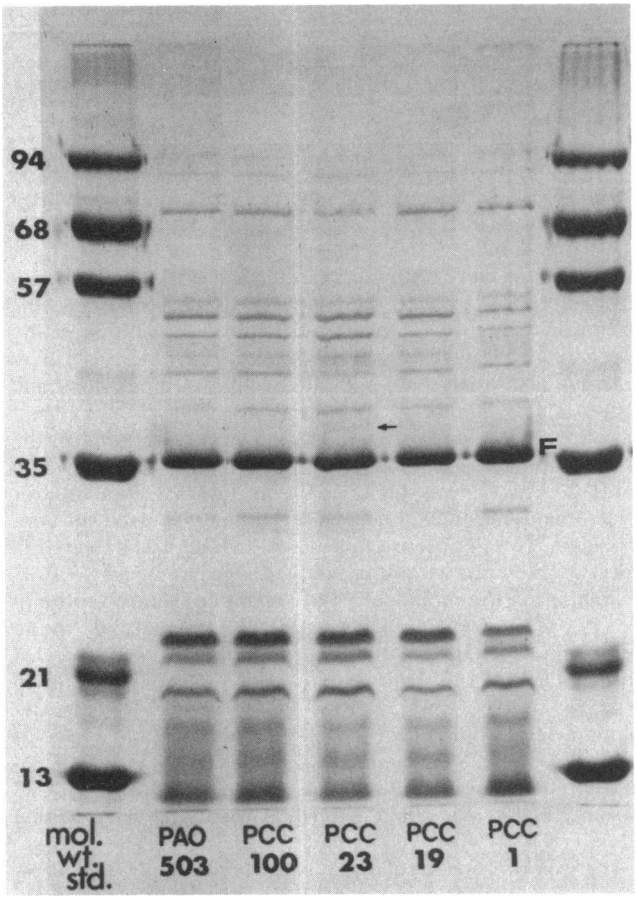

FIG. 1. Polyacrylamide gel electrophoresis of outer membrane proteins isolated from each strain. Outer membrane proteins were obtained as described in the text. The major differences between strains are indicated by the arrow. Porin protein (F) is indicated. Molecular weight standards were phosphorylase B (94,000), bovine serum albumin (68,000), pyruvate kinase (57,000), lactate dehydrogenase (35,000), soybean trypsin inhibitor (21,000), and lysozyme (13,000).

18). The concept of a permeability barrier has also been utilized to explain the high intrinsic resistance to β-lactams (particularly the so-called β-lactamase-resistant β-lactams) in *P. aeruginosa*. The work of Zimmerman (31) with supersusceptible mutants of *P. aeruginosa* has provided evidence for this proposal. Permeability data for the strains examined here (Table 2) show that an additional permeability barrier, resulting in elevated resistance, can arise in *P. aeruginosa*.

The nature of this enhanced barrier is associated with changes in the composition of LPS, since no other significant differences could be detected in the outer membranes of the mutant strains. Changes in the permeability coefficient such as those shown in Table 2 indicate a significant decrease in penetration of the β-lactams (at the concentrations examined) into the cells. Angus et al. (1) suggest that the functional state of hydrophilic pores through the outer membrane can be influenced by the surrounding LPS molecules. The mutant examined by these authors was hypersensitive to β-lactam antibiotics and was proposed to have 5 to 10 times more pores in an open configuration. A larger number of pores in a closed configuration, which would be predicted to increase the resistance level, could result in the resistance observed here.

The patterns of increased MIC (Table 1) indicate that other options were observed here. PCC1 and PCC19, for both penam and cephem antibiotics, required a greater fold increase in MIC of the more hydrophilic antibiotics (low R_m values) than that observed for the less hydrophilic molecules. This trend was more pronounced for the cephem antibiotics, with a clear demarcation between cefotaxime (R_m, 0.19) and ceftriaxone (R_m, 0.25). The demarcation point for penam derivatives was in the vicinity of azlocillin (R_m, 0.86). Nikaido et al. (20) have shown in *E. coli* that permeation of cephems is enhanced when the degree of negativity of the β-lactam is reduced. Hence, dipolar ionic cephems penetrate better than their monobasic analogs. Although strict analogs were not used here, the charge on the molecules cannot be correlated to a similar increase in MIC (compare cefsulodin and ceftazidime for PCC1 and PCC19 in Table 1). The most obvious common difference between PCC1, PCC19, and the parent was the presence of higher than trace amounts of mannose in the LPS profile (Table 3). Mannose has not been described in the LPS of *P. aeruginosa* PAO (13), and its observation here indicates a change, probably within the core of the LPS, since the levels of both fucosamine and the mannuronic acid derivatives were similar to or higher than those found in PAO503. The mannose level in PCC1 was significantly higher, whereas that in PCC19 was only marginally increased. PCC19 also had an elevated level of alanine (another core component), again indicating changes within the core.

Electrical charge was important in the permeation of β-lactams into PCC23 and PCC100 (Table 1). Comparison of the MIC increases for cephems displayed a higher increase for antibiotics containing only negative charges than was observed for the dipolar ionic molecules, cefsulodin and ceftazidime. Nikaido and Rosenberg (19) observed a decrease in permeability concomitant with the addition of a negative charge on the cephem nucleus. The increased resistance of PCC23 and PCC100 to cephem antibiotics is proposed to be the result of enhancement of this barrier function, assuming that the behavior of porin channels in *P. aeruginosa* is similar to that in *E. coli*.

The response of PCC23 and PCC100 to penam antibiotics (Table 1) showed a different behavior. The increased negativity in ticarcillin and Cb resulted in better penetration (lower increase in MIC) than that observed for monobasic penams. This apparent reversal may be negated if the hydrophilic character of the antibiotics is also considered. In the cephems, the R_m value ranged from 0.07 to 0.5, a change of only 0.43. For the penams, this change was considerably greater (1.67), with the largest jump (R_m, 1.27) occurring between Cb (dibasic) and azlocillin (monobasic). Such a difference in the relative hydrophobicities of the penams may counteract the charge considerations. Thus, penetration of the dibasic penams may be unimpeded in a porin channel with increased selectivity for hydrophilic molecules, despite selection against their relatively high negative charge.

Changes in the LPS of PCC23 (Table 3) included an elevated level of fucosamine, indicating an increase in side chain material. Since increasing side chain would increase the hydrophilic domain of the LPS (12), it is unlikely that this would decrease the permeability of hydrophilic β-lactam antibiotics. We have preliminary data suggesting that the function of the porin is changed in PCC23; although a molecular weight change was not involved (Fig. 1), an additional minor protein band was observed in outer membrane profiles.

PCC100, with a resistance phenotype similar to that of

TABLE 3. Composition of assayed components in the LPS

Component	% (Dry wt) in strain[a]:				
	PAO503	PCC1	PCC19	PCC23	PCC100
Rhamnose	1.48 ± 0.14	1.84 ± 0.17	1.93 ± 0.18	2.01 ± 0.23	1.62 ± 0.17
Mannose	Tr[b]	0.8 ± 0.14	0.31 ± 0.04	Tr	–[c]
Galactose	Tr	0.52 ± 0.07	–	Tr	Tr
Glucose	3.11 ± 0.35	5.58 ± 0.57[d]	4.54 ± 0.55	3.92 ± 0.49	3.23 ± 0.57
Galactosamine	2.79 ± 0.28	3.01 ± 0.62	3.01 ± 0.01	2.97 ± 0.23	1.23 ± 0.19
Glucosamine	2.08 ± 0.35	1.59 ± 0.36	1.93 ± 0.11	1.66 ± 0.21	1.81 ± 0.35
Fucosamine	2.18 ± 0.21	2.02 ± 0.19	1.94 ± 0.31	4.39 ± 0.27[d]	1.08 ± 0.36[d]
Mannuronic acid derivatives	2.76 ± 0.25	+[e]	1.97 ± 0.30	3.19 ± 0.41	1.92 ± 0.17
Alanine	1.97 ± 0.30	1.29 ± 0.21	5.02 ± 0.52[d]	1.78 ± 0.33	3.36 ± 0.37[d]
KDO	2.9 ± 0.4	3.2 ± 0.7	3.1 ± 0.4	3.6 ± 0.5	2.2 ± 0.4
Phosphate	15.9 ± 1.62	12.5 ± 0.87	15.9 ± 1.09	7.3 ± 1.12[d]	14.7 ± 1.84

[a] Means ± standard deviation from three preparations assayed on the same column with the same detector settings.
[b] Tr, Trace (<50 pmol in samples, e.g., <0.002% [dry weight] for mannose).
[c] –, Not detected.
[d] Statistically different from PAO503 by the Student *t* test ($P < 0.005$).
[e] +, Present, but not determined.

PCC23, had a very different LPS profile (Table 3). In this case, the side chain sugar fucosamine was markedly reduced, indicating a semirough phenotype. However, PCC100 was also changed in the percentage of alanine, a "core" component. The cumulative effect of these changes upon the nature or function or both of the outer membrane is difficult to predict. The major MIC increases, as indicated earlier, occurred for the more hydrophobic mono-anionic penams and for mono-anionic and dianionic cephems (Table 1). The lower molecular weights of Ticarcillin and Cb (Table 1) may be the governing factor in the relatively small increase in resistance observed here. Penetration of Cb (Table 2) at low concentrations of the β-lactam into PC100 was similar to that observed for PAO503.

We have shown in this study that permeability barriers to β-lactam antibiotics can change in *P. aeruginosa* PAO. Permeability barriers to β-lactam antibiotics have been observed in clinical isolates of *P. aeruginosa* (23). The specific changes within the clinical isolates are unknown. The results with mutants investigated in this study indicate that subtle changes within the composition of the LPS are correlated with increased MICs of β-lactam antibiotics. The two mutants with changes only in the "core" fraction of the LPS resulted in discrimination of the antibiotics based upon their relative hydrophobicities. These changes must occur in the more distal sections of the core, since heptose and KDO (Table 3) percentages were not altered. The two mutants changed within the side chain structure (as well as other changes) resulted in discrimination based upon molecular weight for penams and upon net charge for cephems.

It is probably inadvisable to discuss changes in LPS composition as a sole governing factor in β-lactam permeation, since the porin channel itself has great discriminatory powers (20), and in the native situation, interaction of all outer membrane components (1, 12) contributes to penetration or exclusion. PCC23 illustrates this point. Changes within the LPS imply greater susceptibility to hydrophilic molecules, but a functional change within the porin (unpublished data) altered this predicted phenotype. Similarly, the structural core of the β-lactam may influence its interaction with molecules it must diffuse through to reach the periplasm. Whether the type or state of LPS can influence the open-closed state of pores, as suggested by Angus et al. (1), or whether a change in the hydrophilic-hydrophobic nature of the outer layers of the cell—either generally or at specific sites—results in altered permeation, remains to be tested. The observation that LPS changes are correlated with decreased permeability and increased resistance in *P. aeruginosa* implies a major role for this layer in resistance to β-lactam antibiotics.

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