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Penetration of β-Lactam Antibiotics into Their Target Enzymes in *Pseudomonas aeruginosa*: Comparison of a Highly Sensitive Mutant with Its Parent Strain

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Pseudomonas aeruginosa K 799/WT and a mutant of this strain, *P. aeruginosa* K 799/61 ("mutant 61"), that is very sensitive to most β-lactam antibiotics tested were used to assess the importance of penetration barriers in the resistance of *P. aeruginosa* to penicillins and cephalosporins. The affinities of various β-lactams to the penicillin-binding proteins found in membranes prepared from both strains were compared by measuring their competition for the binding of benzyl[^{14}C] penicillin to each of these proteins. Only minor differences between the wild type and the mutant 61 were found. The high sensitivity of the mutant therefore cannot be attributed to drastic alterations of these target proteins, nor can the resistance of the wild type be ascribed to penicillin-binding proteins with low affinities for β-lactams. Experiments in which the ease of penetration of β-lactams into the penicillin-binding proteins was measured with exponentially growing intact cells instead of membranes, however, clearly demonstrated an easy access of β-lactam antibiotics to these proteins in the mutant and an efficient exclusion from the same targets in the wild type.

Pseudomonas aeruginosa is one of the few clinically important pathogens resistant to a large number of antibiotics otherwise active against a broad spectrum of bacteria (3). A large proportion of these strains certainly owe their resistance to enzymes that inactivate or modify antibiotics. Very little, however, is known of the extent to which the resistance is also due to efficient penetration barriers. In an effort to obtain more insight into this type of resistance, we began a search for mutants of *P. aeruginosa* showing increased sensitivity towards a large number of antibiotics with different modes of action, closely analogous to work done with *Escherichia coli* by Richmond et al. (9). One of the mutants isolated showed an unusually high sensitivity to β-lactam antibiotics. The antibacterial activities of various antibiotics against this mutant have already been described (13). In this work the affinities of several β-lactam antibiotics for the penicillin-binding proteins (PBPs) of the mutant and the wild type are compared. Direct evidence is given that β-lactam antibiotics can be efficiently excluded from their target enzymes in the wild type but have nearly unhindered access to the same proteins in the mutant.

MATERIALS AND METHODS

Bacterial strains, growth conditions, media, and buffers. *P. aeruginosa* K 799/WT (the wild type, ATCC 12055) and its mutant *P. aeruginosa* K 799/61 ("mutant 61") were used in all experiments. They were grown in Penassay broth (Difco antibiotic medium no. 3) at 37°C. Membrane buffer was 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$.

Mutant isolation. Mutagenesis induced with ethyl methane sulfonate was performed as described by Mills and Holloway (8); mutagenesis induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was according to the procedure described by Adelberg et al. (1). The mutagenized cultures were grown overnight to allow for segregation and then suitably diluted to give single colonies on agar plates. These colonies were then replica plated on plates containing antibiotic. Colonies not growing on these were picked from the master plate and characterized further. The final selection of the mutant 61 was on plates containing cephalosporin C (0.5 μg/ml).

Measurement of MICs. Minimal inhibitory concentrations (MICs) were determined by the agar plate dilution method (6). Log-phase cultures in brain heart infusion broth were suitably diluted and inoculated by a multiple inoculator (11) on brain heart infusion agar. The inoculum consisted of about 10^4 colony-forming units.

Preparation of membranes. The bacteria were grown under vigorous aeration to a density of about 5 × 10^8 cells/ml. After cooling on ice, they were centrifuged at 6,000 × *g* for 10 min, and the cell sediments from 600 ml of culture were resuspended in 100 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$. The cells were broken at 0°C by six 30-s pulses of sonication with intervening 15-s periods of cooling (Branson B-12 Sonifier, Branson Sonic Power Co., Danbury, Conn.). The resulting suspensions were centrifuged at 100,000 × *g* for 45 min at 2°C in a Beckman ultracentrifuge, the sedimented

membranes were suspended in 60 ml of the same buffer, and the ultrasonic treatment as well as the centrifugation were repeated as above. The membranes were finally suspended in membrane buffer, adjusted to 5 to 10 mg of protein per ml, and stored at −80°C.

Binding of β-lactams to the PBPs in membranes of the wild type and the mutant 61. The binding of six nonradioactive β-lactams was studied by measuring their competition for the binding of benzyl[^{14}C]penicillin to each of the PBPs of the wild type and the mutant 61. The procedure followed essentially that described by Spratt (10). A 90-μl volume of membranes was preincubated with 10 μl of either buffer or dilutions of the nonradioactive β-lactam for 10 min at 37°C, and then 10 μl of 1.1×10^{-4} M benzyl[^{14}C]penicillin was added for a further 10 min at 37°C. The binding was terminated by the addition of 5 μl of nonradioactive benzylpenicillin (120 mg/ml) and 10 μl of 10% (wt/vol) Sarkosyl. After 20 min of standing at room temperature, the Sarkosyl-insoluble outer membrane and peptidoglycan were removed by centrifugation at 40,000 × *g* for 60 min at 15°C. A 100-μl volume of the Sarkosyl-soluble supernatant was added to 50 μl of gel sample buffer [0.2 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8)-3% (wt/vol) sodium dodecyl sulfate (SDS)-30% (vol/vol) glycerol-0.003% (wt/vol) bromophenol blue]. A 15-μl volume of 2-mercaptoethanol was added to each sample immediately before they were heated for 2 min in a boiling water bath. These samples were then used for the separation of the PBPs by SDS-polyacrylamide slab gel electrophoresis.

Penetration and binding of β-lactams to the PBPs in intact cells of *P. aeruginosa*. Cultures of *P. aeruginosa* K 799/WT or the mutant 61 were grown under vigorous aeration to a density of about 5×10^8 bacteria/ml and centrifuged without cooling at 6,000 × *g* for 10 min, and the resulting sediment from 100 ml of culture was suspended at room temperature in 2 ml of membrane buffer. Portions (100 μl) of this suspension were preincubated in a water bath for 5 min to 37°C; 10 μl of benzyl[^{14}C]penicillin at various concentrations was added, and the incubation continued for 5 min. Further binding of benzyl[^{14}C]penicillin was terminated by adding 10 μl of nonradioactive benzylpenicillin (120 mg/ml) and the cells were broken by ultrasonic treatment in a sonication bath (50 kHz, Elgasonic, Elga SA, Bienne, Switzerland). The broken cells were chilled immediately on ice and centrifuged at 40,000 × *g* for 20 min at 2°C in a Sorvall RC-5B centrifuge to separate membranes from cytoplasmic proteins. The resulting sediments were suspended in 150 μl of threefold-diluted gel sample buffer (see above), and the PBPs were separated by SDS-polyacrylamide slab gel electrophoresis. Identical experiments in which cells from the same washed cell suspension were first disrupted by ultrasonic treatment before they were exposed to benzyl[^{14}C]penicillin were always run in parallel for direct comparison.

The ease of penetration of nonradioactive β-lactams was measured in the following way: to cultures of the wild type or the mutant 61 (5 ml, about 5×10^8 cells/ml) growing under vigorous aeration as above, cefsulodin or cefoxitin were added to give a final concentration of 1 μg/ml. The cultures were grown under identical conditions for a further 5 min and then rapidly chilled on ice and centrifuged at 6,000 × *g* for 5 min at 2°C. The pelleted cells were washed once with 5 ml of ice-cold membrane buffer to remove free antibiotic, and the centrifugation was repeated. The washed cell pellets were suspended in 100 μl of the same buffer, and the cells were disrupted by ultrasonic treatment. Residual binding of benzyl[^{14}C]penicillin was measured by adding 10 μl of benzyl[^{14}C]penicillin (1.1×10^{-4} M) for 5 min at 37°C. Further binding was prevented, and the samples were prepared for SDS-polyacrylamide slab gel electrophoresis as described above.

The results obtained from these measurements were compared with those from analogous experiments in which broken cell suspensions instead of intact cells were preexposed to cefsulodin or cefoxitin. The washed cell pellets obtained from 5 ml of cultures grown as described above were suspended in 90 μl of membrane buffer, and the cells were disrupted by ultrasonic treatment in a sonication bath. The resulting broken cell suspensions were prewarmed to 37°C, and 10 μl of cefsulodin or cefoxitin (1 μg/ml, final concentration) was added for 5 min followed by 10 μl of benzyl[^{14}C]penicillin (10^{-5} M, final concentration) for 5 min. Further binding was prevented by adding 10 μl of nonradioactive benzylpenicillin (120 mg/ml). The suspensions were then chilled immediately on ice and centrifuged at 40,000 × *g* for 20 min at 2°C, and the resulting sediments were prepared for SDS-polyacrylamide slab gel electrophoresis. In all of these experiments, controls without cefsulodin or cefoxitin were always run in parallel.

SDS-polyacrylamide slab gel electrophoresis and detection of PBPs. The procedure for SDS-polyacrylamide slab gel electrophoresis was that of Laemmli and Favre (7) with modifications as described by Spratt (10). The running gel was composed of 10% (wt/vol) acrylamide with a ratio of acrylamide to *N*,*N*'-methylenebisacrylamide of 100 to 1. Electrophoresis was carried out at a constant current of 25 mA for 4 to 5 h. The gels were prepared for fluorography, and the PBPs were detected by contacting the dried gel with Kodak RP Royal X-Omat X-ray film at −70°C as described by Spratt (10). Exposure of the gel to the X-ray film was for 4 to 6 weeks. Quantitation of the levels of benzyl[^{14}C]penicillin bound to the PBPs was carried out by microdensitometry (Quick Scan densitometer, Helena Laboratories, Beaumont, Tex.).

Chemicals. Benzyl[^{14}C]penicillin was purchased from the Radiochemical Centre, Amersham, England (specific activity, 53 to 54 Ci/mol). Cefsulodin (12) is a semisynthetic antipseudomonal cephalosporin codeveloped by Takeda Chemical Industries, Ltd., Osaka, Japan, and Ciba-Geigy Ltd., Basel, Switzerland. The other β-lactam antibiotics investigated were carbenicillin (Beecham Research Laboratories, Brentford, England), azlocillin (Bayer AG, Leverkusen, Germany), cephaloridine (Glaxo Research Ltd., Greenford, England), cephalexin (Eli Lilly & Co., Indianapolis, Ind.), and cefoxitin (Merck, Sharp and Dohme, Rahway, N.J.). SDS (lauryl sulfate no. L-5750) was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals used for SDS-polyacrylamide slab gel

electrophoresis were purchased from Serva Feinbiochemica GmbH & Co., Heidelberg, Germany. Sodium lauroyl sarcosinate (Sarkosyl NL-97) was from Ciba-Geigy Ltd., Basel, Switzerland.

RESULTS

General properties of the mutant 61. The mutant 61 was obtained through four-step mutagenesis induced with ethyl methane sulfonate and a final treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (see Materials and Methods). Compared with the wild type, it showed a general increase in antibiotic susceptibility and was unusually sensitive to all β-lactam antibiotics tested (13). In complete media it grew nearly as well as the wild type. There were no significant differences between the mutant and the wild type in the pattern of their membrane proteins. The exact biochemical lesion of the mutant has not yet been characterized.

Binding of β-lactam antibiotics to the PBPs in membranes prepared from *P. aeruginosa* K 799/WT and the mutant 61. It is now widely accepted that bacterial cells contain a number of different penicillin-sensitive enzymes, and that penicillin inhibits these enzymes by forming a stable penicilloyl-enzyme complex (2). The penicillin-sensitive enzymes can be detected as those proteins that covalently bind radioactive penicillin. An assay for penicillin-binding proteins therefore provides a convenient method for studying the properties of the penicillin-sensitive enzymes of a bacterium in their normal membrane environment (10). Alterations in the sensitivities of one or several penicillin-sensitive enzymes might be a simple explanation for the large differences observed in the sensitivities of *P. aeruginosa* K 799/WT and its mutant 61 to β-lactam antibiotics. We therefore compared the affinities of several β-lactam antibiotics for each of the PBPs in the wild type and the mutant 61. Figure 1 shows the numbering of the PBPs used throughout and the results obtained with both strains in a typical competition experiment. It is quite clear from these data that the wild type and the mutant 61 possess the same set of PBPs and that the affinity of cephaloridine for each of the PBPs in both strains is the same. PBP 2 was not detected under these experimental conditions (10^{-5} M benzyl[^{14}C]penicillin). A numerical comparison of the binding of six β-lactams to the PBPs of the wild type and the mutant 61 is given in Table 1. With both strains the concentrations of β-lactam required to reduce benzyl[^{14}C]penicillin binding by 50% were practically the same. The small differences observed are certainly negligible when one compares them with the very large differences between the MICs of both strains given in the last column of the same Table. The resistance of wild-type *P. aeruginosa* against many β-lactam antibiotics is therefore certainly not due to insensitivity of the bacterial target enzymes. The data given in Table 1 also show that the MICs of the six antibiotics against the mutant 61 compare well with the low concentrations needed to compete with benzyl[^{14}C]penicillin binding, cephalexin being an exception.

Penetration of benzyl[^{14}C]penicillin to the PBPs in intact cells of *P. aeruginosa*. The strong similarities between the PBPs of the wild type and the mutant found in the preceding experiments made it probable that differences in target accessibility might be the main reason for the large differences observed in the susceptibility of these two strains to β-lactam antibiotics. To study the ease of penetration of benzyl-[^{14}C]penicillin to the PBPs of the wild type and the mutant 61, binding experiments using intact cells were done. The results were compared with those from experiments with broken cell suspensions prepared from the same cultures by ultrasonic treatment, assuming unhindered access of benzyl[^{14}C]penicillin to the PBPs under this condition. The results obtained with the wild type and the mutant 61 are given in Fig. 2 and 3, respectively. These experiments clearly demonstrated a nearly unhindered penetration of benzyl[^{14}C]penicillin to the PBPs in intact cells of the mutant 61. On the other hand, when one compares the results obtained with intact cells to those obtained with broken cell suspensions, the existence in the wild type of an efficient penetration barrier for the same compound is obvious.

The efficient labeling of PBP 4 seen in the experiments with intact cells of the wild type does not mean that the barrier is inoperative for this PBP. Benzylpenicillin has an extremely high affinity for this protein. In experiments with broken cell suspensions, PBP 4 is fully saturated at 1 μM benzyl[^{14}C]penicillin (compare lanes B and F in Fig. 2), and it becomes labeled at concentrations as low as 0.01 μM (data not shown). In the experiment with intact cells of the wild type, the amount of radioactivity bound to PBP 4 at 100 μM benzyl[^{14}C]penicillin (Fig. 2, lane A) was smaller than in the experiment with a broken cell suspension at only 1 μM concentration (lane F). This clearly means that the barrier is working efficiently for PBP 4 too. A similar comparison of the labeling of PBP 5, however, does not exclude a differential accessibility of some of the PBPs in the cytoplasmic membrane of the intact cell.

Penetration of cefsulodin and cefoxitin through the outer cell wall layers of *P. aeruginosa* K 799/WT and the mutant 61.

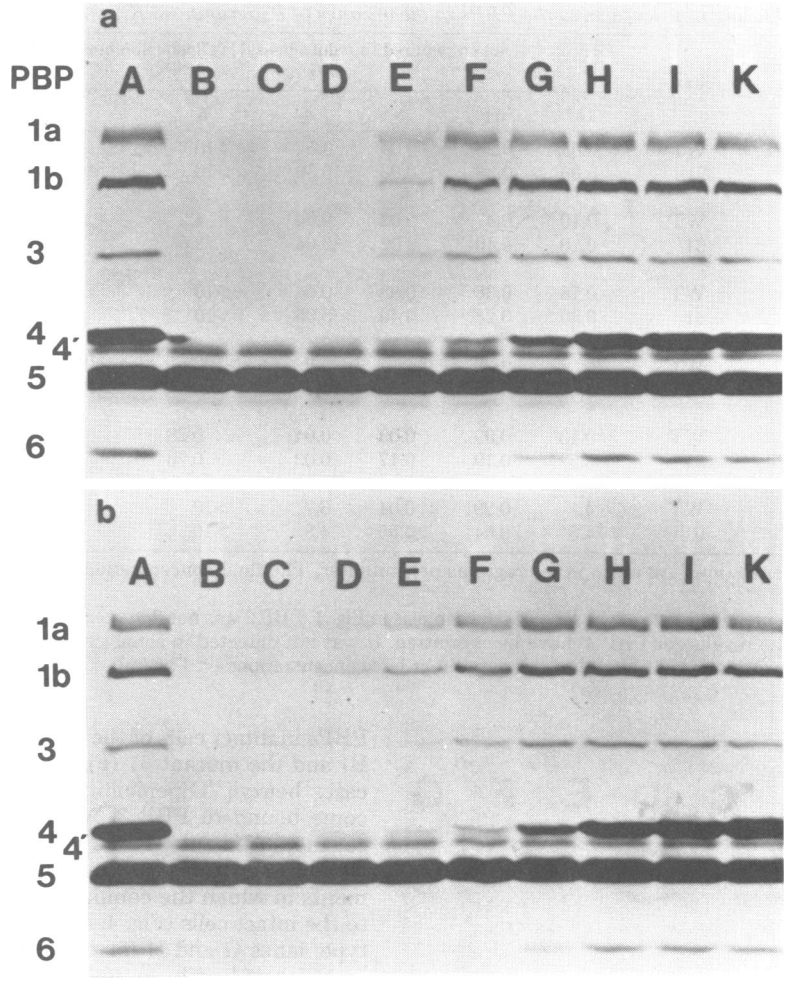

FIG. 1. *Numbering of the PBPs of P. aeruginosa and competition of cephaloridine for benzyl[^{14}C]penicillin binding to the PBPs of P. aeruginosa K 799/WT (a) and the mutant 61 (b). The final concentrations of the competing cephaloridine were (in micrograms per milliliter): (A, K) 0, (B) 30, (C) 10, (D) 3, (E) 1, (F) 0.3, (G) 0.1, (H) 0.03, (I) 0.01. For experimental details see the text.*

The experiments described above clearly demonstrated large differences between the wild type and the mutant 61 with regard to the penetration of benzylpenicillin into the PBPs in the cytoplasmic membrane. It was therefore interesting to know whether other β-lactam antibiotics with equally good activity against the mutant 61, but with significant differences in their activity against the wild type, would also show a corresponding difference in their ease of penetration through the outer layers of the wild type. We therefore measured residual binding of benzyl[^{14}C]penicillin to the PBPs in broken cell suspensions prepared by ultrasonic treatment from log-phase cultures of the wild type and the mutant 61 grown for 5 min in the presence of 1 μg of cefsulodin or cefoxitin per ml. Cefsulodin was selected because, at a concentration of 1 μg/ml, it inhibits the growth of both the wild type and the mutant 61. Cefoxitin, on the other hand, is inactive against the wild type, but nearly as active as cefsulodin against the mutant 61 (see the last column of Table 1). Analogous experiments in which the two cephalosporins were added not to the growing bacteria but to the broken cell suspensions prepared from identical cultures were always run for comparison. Again the assumption was made that the two cephalosporins as well as benzyl[^{14}C]penicillin had free access to the PBPs in a broken cell suspension. The results of these experiments are given in Fig. 4. Since they were performed in four groups (see legend to Fig. 4), the amount of benzyl-[^{14}C]penicillin bound to the individual PBPs

TABLE 1. *Binding of β-lactams to the PBPs in membranes of P. aeruginosa K 799/WT and its mutant 61*

β-Lactam antibiotic	Strain	Concn of β-lactam required to reduce benzyl [^{14}C]penicillin binding by 50%[a]						MIC (μg/ml)
		1A[b]	1B	3	4	5	6	
Carbenicillin	WT	0.08	0.26	0.04	0.54	>10	1.6	8
	61	0.06	0.38	0.03	0.50	>10	1.5	0.02
Azlocillin	WT	0.10	0.47	0.02	0.04	4.2	0.08	2
	61	0.10	0.40	0.02	0.04	3.6	0.08	0.01
Cephaloridine	WT	0.78	0.38	0.88	0.04	>10	0.10	>128
	61	0.30	0.28	0.40	0.05	>10	0.11	0.5-1
Cephalexin	WT	35	0.73	41	0.9	>100	>100	>128
	61	38	0.78	36	1.7	>100	>100	16
Cefoxitin	WT	0.13	0.09	0.09	0.04	0.28	0.05	>128
	61	0.22	0.19	0.17	0.03	0.26	0.03	0.05
Cefsulodin	WT	4.1	0.29	0.04	6.7	>10	14	0.5
	61	4.5	0.64	0.05	4.8	>10	9	0.03

[a] The concentrations are given in micrograms per milliliter. The final concentration of benzyl[^{14}C]penicillin was 10^{-5} M.

[b] PBP. The nomenclature of the PBPs is the same as in Fig. 1. PBP 2 was not detected under our experimental conditions. The results for PBP 4' have been omitted. It was not detected in some experiments, and cefoxitin was the only antibiotic that bound to this protein at low concentrations (< 1 μg/ml).

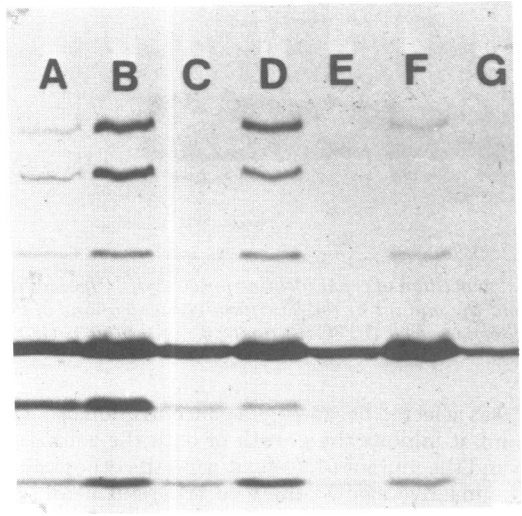

FIG. 2. *Binding of benzyl[^{14}C]penicillin to the PBPs in intact cells and broken cell suspensions of P. aeruginosa K 799/WT. (A, C, E, G) Intact cells; (B, D, F) broken cells prepared from the same washed cell suspension by ultrasonic treatment. The final concentrations of benzyl[^{14}C]penicillin were: (A) 100, (B, C) 10, (D, E) 3, and (F, G) 1 μM. For experimental details see the text.*

should always be compared with that of the corresponding control experiment without cefsulodin or cefoxitin. With cefsulodin there were only small differences in the accessibility of the PBPs in intact cells of the wild type (Fig. 4, lane B) and the mutant 61 (Fig. 4, lane H). In both cases benzyl[^{14}C]penicillin could no longer become bound to PBP 3. A good penetration of cefsulodin into the PBPs in both strains could also be seen from a comparison of the experiments in which the competitor had been added to the intact cells (Fig. 4, lanes A and B for wild type; lanes G and H for mutant 61) with those in which it had been added to the broken cell suspensions prepared from the same strains (Fig. 4, lanes D and E for wild type; lanes K and L for mutant 61). In contrast to the results obtained with cefsulodin, the existence in the wild type of a marked penetration barrier for cefoxitin was evident (compare lanes C and F, Fig. 4): a comparison with the corresponding control (lane A) showed that preexposure of a log-phase culture of the wild type to cefoxitin did not reduce the amount of benzyl[^{14}C]penicillin that subsequently was still bound to the PBPs 1A, 1B, and 5. In the corresponding experiment with the mutant 61, however, the amount of benzyl-[^{14}C]penicillin bound to the same PBPs was only 35, 23, and 13%, respectively, of that bound in the control without preexposure to cefoxitin (compare lanes G and I, Fig. 4).

DISCUSSION

The results described in this paper with *P. aeruginosa* K 799/WT and its mutant 61 clearly show that inadequate penetration through the

cell envelope is a significant factor in the resistance of *P. aeruginosa* to β-lactam antibiotics. With reference to their PBPs, *P. aeruginosa* K 799/WT and the mutant 61 cannot be distinguished from each other. The large differences in antibacterial activities found with penicillins and cephalosporins against these two strains, however, can be explained by the existence of a penetration barrier in the wild type which is much reduced in the mutant 61. It is interesting to note that the efficiency of this barrier is much more pronounced in *P. aeruginosa* than in *E. coli* (4, 5). The data also show that the ease of penetration of a β-lactam antibiotic to its target enzymes is very much influenced by its chemical structure. The parameters required for good penetration through the outer layers of the *P. aeruginosa* cell envelope, however, are still largely unknown.

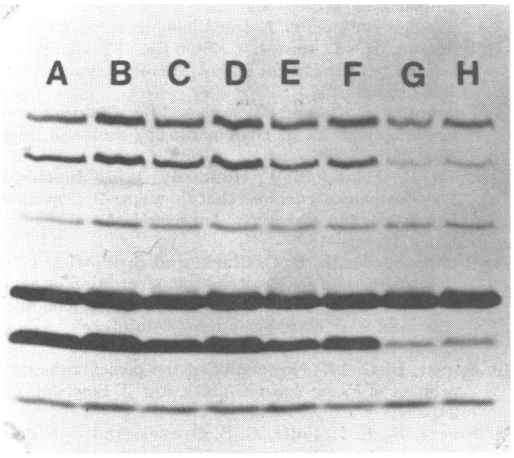

FIG. 3. *Binding of benzyl[^{14}C]penicillin to the PBPs in intact cells and broken cell suspensions of the mutant 61. (A, C, E, G) Intact cells; (B, D, F, H) broken cells prepared from the same washed cell suspension by ultrasonic treatment. The final concentrations of benzyl[^{14}C]penicillin were: (A, B) 100, (C, D) 10, (E, F) 3, and (G, H) 1 μM. For experimental details see the text.*

The mutant 61 is a true permeation mutant. It allows nearly unhindered access of most β-lactam antibiotics to the PBPs in the inner membrane. The chemical basis of the lesion in the mutant cell envelope is still unknown. A comparison of the membrane proteins found in the wild type and the mutant 61 by SDS-polyacrylamide slab gel electrophoresis revealed no obvious differences between these two strains (data not shown). Whatever its structural basis, the mutant 61 can be useful for measuring the activity of β-lactams under conditions where permeability barriers between the antibiotics and their targets are negligible. A comparison of the MICs of β-lactam antibiotics against *P. aeruginosa* K 799/WT and the mutant 61 allows one to evaluate penetrability and "intrinsic" activity simultaneously by one simple test.

It should finally be pointed out that the experiments were not aimed at drawing conclusions on the importance of the individual PBPs

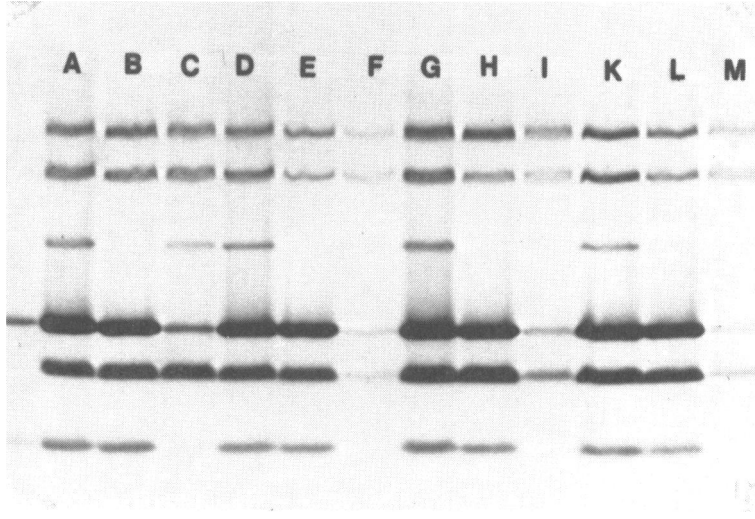

FIG. 4. *Penetration of cefsulodin and cefoxitin into the PBPs in intact cells of P. aeruginosa K 799/WT and its mutant 61. The experiments were performed in four groups as described in the text: (A–C) P. aeruginosa K 799/WT, competitor added to exponentially growing intact cells; (D–F) competitor added to the broken cell suspensions prepared from the same strain by ultrasonic treatment; (G–I) mutant 61, intact cells; (K–M) broken cell suspensions of the same strain. (A, D, G, K) Controls, without competitor; (B, E, H, L) cefsulodin, 1 μg/ml; (C, F, I, M) cefoxitin, 1 μg/ml.*

for the inhibition and killing of *P. aeruginosa* by penicillins and cephalosporins. We just used the identical affinities for β-lactams of the PBPs found in both strains and their localization in the cytoplasmic membrane to show differences between the wild type and the mutant 61 with regard to their penetrability of β-lactam antibiotics. Since in the competition experiments with intact cells of the wild type the concentration of cefoxitin was far below its MIC, it was surprising that binding of this compound to the PBPs 3, 4, and 6 occurred (Fig. 4, lane C). This may imply that inhibition of some or all of these proteins is not lethal for *P. aeruginosa* or that even a small amount of functional protein is sufficient for survival. The same data also do not exclude differences in the accessibility of the individual PBPs. This possibility deserves further investigation.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Cheng.** 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Blumberg, P. M., and J. L. Strominger.** 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. **38**:291–335.
3. **Brown, M. R. W. (ed.).** 1975. Resistance of *Pseudomonas aeruginosa*. John Wiley & Sons, London.
4. **Curtis, N. A. C., C. Brown, M. Boxall, and M. G. Boulton.** 1979. Inhibition of *Escherichia coli* K-12 by β-lactam antibiotics with poor antibacterial activity: interaction of permeability and intrinsic activity against penicillin-binding proteins. Antimicrob. Agents Chemother. **15**:332–336.
5. **Curtis, N. A. C., D. Orr, G. W. Ross, and M. G. Boulton.** 1979. Competition of β-lactam antibiotics for the penicillin-binding proteins of *Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella aerogenes, Proteus rettgeri*, and *Escherichia coli*: comparison with antibacterial activity and effects upon bacterial morphology. Antimicrob. Agents Chemother. **16**:325–328.
6. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing. Acta Pathol. Microbiol. Scand. Sect. B, **79**(Suppl.):217.
7. **Laemmli, U. K., and M. Favre.** 1973. Maturation of bacteriophage T4. 1. DNA packaging events. J. Mol. Biol. **80**:575–599.
8. **Mills, B. J., and B. W. Holloway.** 1976. Mutants of *Pseudomonas aeruginosa* that show specific hypersensitivity to aminoglycosides. Antimicrob. Agents Chemother. **10**:411–416.
9. **Richmond, M. H., D. C. Clark, and S. Wotton.** 1976. Indirect method for assessing the penetration of beta-lactamase-nonsusceptible penicillins and cephalosporins in *Escherichia coli* strains. Antimicrob. Agents Chemother. **10**:215–218.
10. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**: 341–352.
11. **Steers, E., E. L. Foltz, B. S. Graves, and J. Riden.** 1959. An inocula-replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307–311.
12. **Tsuchiya, K., M. Kondo, and H. Nagatomo.** 1978. SCE-129, antipseudomonal cephalosporin: in vitro and in vivo antibacterial activities. Antimicrob. Agents Chemother. **13**:137–145.
13. **Zimmermann, W.** 1979. Penetration through the gram-negative cell wall: a co-determinant of the efficacy of beta-lactam antibiotics. Int. J. Clin. Pharm. Biopharm. **17**:131–134.