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# Antibiotic Diffusion Pathways in the Outer Membrane of *Pseudomonas aeruginosa*

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**We investigated the effect of a temperature shift from 37°C to 17°C on the steady-state diffusion rate of imipenem and cephalothin by evaluating periplasmic drug concentrations in intact cells of *Pseudomonas aeruginosa,* which overexpresses the extended spectrum β-lactamase. We found that the ratio of periplasmic imipenem concentraton at 17 °C relative to that of 37 °C was 1.03±0.1, whereas that of cephalothin was 0.43± 0.09. Accumulation rates of cell-associated tetracycline and fluoroquinolone at 17°C were roughly 1/16 and 1/8, respectively, compared with that at 37°C. We concluded from these data that cephalothin and possibly most other antibiotics excepting carbapenems cross the outer membrane of *P. aeruginosa* mainly by dissolving in the lipid phase but probably not passing through the porin channel. This may explain why the outer membrane of *P. aeruginosa* is a tight barrier against the penetration of antibiotics.** 

Most antibiotics have to enter the target cell by crossing the membrane(s) before exerting their effect. Diffusion of antibiotics into the gram-negative bacteria is particularly complex, since the bacteria consist of double membrane layers. Small hydrophilic molecules, including antibiotics, have been thought to cross the outer membrane through water filled porin channels (see 1, 2 for review). The OmpF channel of *Escherichia coli,* for example, allows the diffusion of saccharides of molecular weight ($M_r$) less than 600 and excludes molecules larger than this threshold (3).

Studies on the outer membrane permeability of *Pseudomonas aeruginosa* have led to two opposing conclusions: (i) the membrane is permeable to the size of polysaccharides with an $M_r$ up to 7,000 and protein F (OprF) is responsible for this channel activity (4); and (ii) a threshold of permeable saccharides with an $M_r$ 350 or less and proteins C, D and E1 are responsible for this channel activity (5, 6). To clarify this discrepancy, we examined the minimum inhibitory concentrations (MIC) of antibiotics such as β-lactams, fluoroquinolones, tetracycline, and chloramphenicol in single deletion mutants lacking *oprC, oprD* or *oprE,* double or triple deletion mutants lacking all combinations of these genes, and the mutant lacking OprF. We found that the MICs of most antibiotics in these mutants were fully comparable with that in the wild type strain (7). The only exception was that the MIC of imipenem in all mutants lacking OprD appeared to be 8 to 16 times higher than that in the wild type parent strain. These results suggest that most antibiotics except for imipenem are unable to diffuse through the porin channels.

The question that has remained unanswered is how most antibiotics excepting carbapenems cross the outer membrane of *P. aeruginosa*. We hypothesized that they might cross the membrane by dissolving in the hydrophobic lipid domain. If so, the diffusion rate of antibiotics, which cross the membrane by dissolving in the lipid bilayer may be largely retarded by lowering the assay temperature, whereas the temperature effect may be minimum for antibiotics that permeate through porin channels. To test this hypothesis, we compared the steady-state diffusion rate of cephalothin at 17°C and 37°C in intact cells harboring the plasmid carrying the extended spectrum β-lactamase gene and the result was compared with that of imipenem, whose diffusion pathway has been firmly established as the OprD porin channel (8). Cell-associated fluoroquinolone, tetracycline and chloramphenicol rates were also determined at 17°C and 37°C.

## MATERIALS AND METHODS

*Bacterial strains and plasmid.* Bacterial strains used were derivatives of *P. aeruginosa*. Relevant properties of the strains were as follows. Strain PAO4260 is an *rec* mutant. TNP004, TNP066 and TNP067 lack OprD, OprC /OprE1 and OprC/OprD/OprE1, respectively (7). TNP076, a derivative of PAO4290, lacks all subunits of the MexA-MexB-OprM antibiotic extrusion pump (9). pMS363 is a plasmid carrying the extended spectrum β-lactamase gene.

*The recombinant DNA techniques.* This was conducted by the standard method described elsewhere (10). The plasmid pMS363 was



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transformed to PAO4260, TNP004, TNP006, TNP067 and TNP076 and the presence of specific restriction fragments of the plasmid DNA was confirmed by agarose gel electrophoresis. Construction of deletion mutant lacking all subunits of the MexA,B-OprM extrusion pump has been described previously (9)

*Permeability assay of β-lactam antibiotics.* Cells grown overnight in 4 ml of L-broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 5 mM $MgCl_2$, 300 mg of CBPC per liter , pH 7.2) were diluted with 30 ml of fresh medium, rotated at 200 rpm for 4 h, harvested by centrifugation at 30°C, suspended in 10 ml of 100 mM NaCl-50 mM Mops buffer pH 7.5-1 μM $ZnCl_2$ (NaCl-Mops buffer), and centrifuged at 7000 × g for 10 min at 30 °C. Cells were gently suspended in NaCl-Mops buffer to 50 mg per ml. Permeability of β-lactams was determined by the Zimmermann and Rosselet method (11). To a 3-ml cuvette, 300 μl of 1 mM substrate and 2685 μl of NaCl-Mops buffer were added and kept in water bath at the desired temperature for 10 min. To this was added 15 μl of cell suspension and the rate of substrate hydrolysis was recorded at 299 and 262 nm for imipenem and cephalothin, respectively, by a Hitachi spectrophotometer 200-20 connected to a temperature controlled circulating water bath. β-lactamase activity in cell-free extracts was also determined. A cuvette holder was maintained at the desired temperature throughout.

*Determination of the cell associated antibiotics.* Strain TNP076 was grown in 10-ml of L-broth at 37°C overnight, diluted with 200 ml of fresh medium, rotated at 200 rpm at 37°C for 4 h and harvested by centrifugation. Cells were suspended in 20 ml of 100 mM NaCl-20 mM Na-$PO_4$ buffer (NaCl-$PO_4$ buffer) and centrifuged again at 7000 × g for 10 min at 25°C. Cells were suspended to 50 mg per ml in a solution of 100 mM NaCl-$PO_4$ buffer-0.05% glycerol. To a 1.5-ml Eppendorf centrifuge tube was added 0.4 ml of the cell suspension, incubated at the desired temperature for 10 min, and 10-μl aliquot of 4 mg (or 0.4 mg) per ml of antibiotic was added and incubated at the desired temperature. The tube was centrifuged at 14,000 rpm for 2 min at 4°C by an Eppendorf centrifuge, then the pellet was suspended in 1 ml of 100 mM NaCl-20 mM $PO_4$ buffer and centrifuged again at 14,000 rpm at 4°C for 2 min. Cell-associated tetracycline, ciprofloxacin and [$^{14}$C]-chloramphenicol were quantified by determining absorption at 440 nm, fluorescence emission at 450 nm by excitation wave length at 285 nm and by counting the radioisotope, respectively.

## RESULTS

### *Comparison of the Diffusion Rates of Cephalothin and Imipenem at 37 °C and 17 °C*

We hypothesized that the effect of assay temperature on the diffusion rate of imipenem might be small as it mainly crosses the outer membrane through the OprD channel, whereas that of cephalothin might be large if it crosses the outer membrane dissolving in the lipid domain. We set the assay system to monitor the rate of imipenem or cephalothin hydrolysis by the periplasmic β-lactamase encoded by the extended spectrum β-lactamase gene. The Kms of the β-lactamase to imipenem and cephalothin were reported to be 24.6 and 6.1 μM, respectively, and the relative Vmaxs were 116 and 113, respectively (12).

In the first experiment, we determined the relative diffusion rate (17°C/37°C) of cephalothin and imipenem in three strains producing a wild type level of OprD (Fig. 1A). Cell-free enzyme activity at 17°C was 36 to 38% of that at 37°C. The periplasmic imipenem concentrations at 17°C and 37°C were calculated incorporating Km and Vmax of the enzyme to the respective substrates according to the Zimmermann and Rosselet, and found to be an average 3.0 and 2.9 μM, respectively at 100 μM of external substrate. The ratio of periplamic imipenem concentration at 17°C relative to that at 37°C appeared to be 1.03±0.1 suggesting that the diffusion rate of imipenem was merely influenced by the assay temperature, thereby confirmed that imipenem crosses the outer membrane by dissolving in the water phase through the OprD channel (Fig. lA). Similar experiments were conducted using the same strains for cephalothin at 17°C and 37°C and the periplasmic drug con-

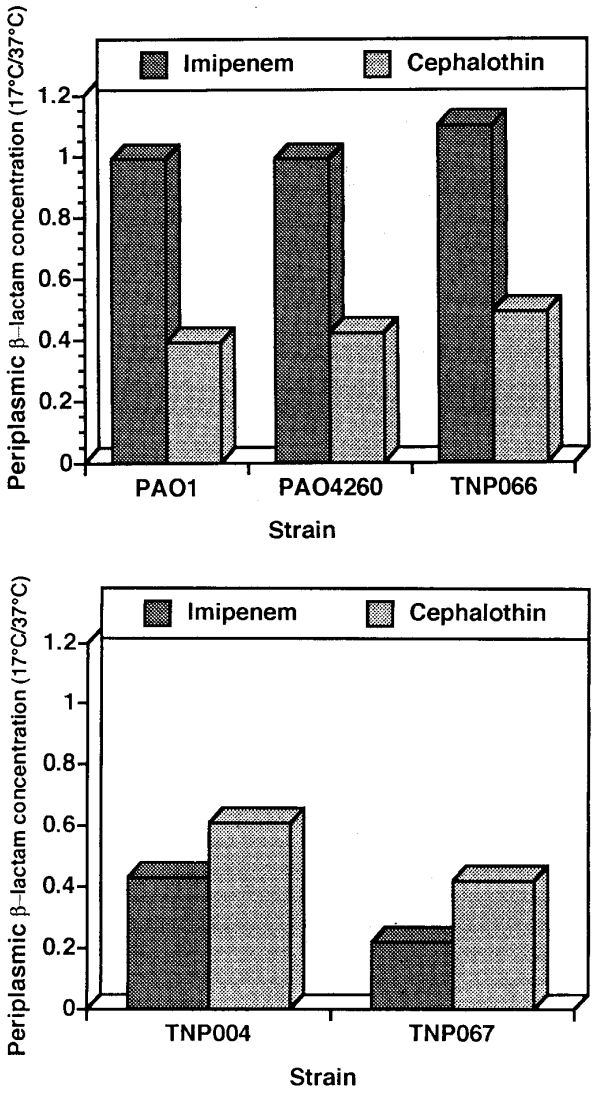


**FIG. 1.** Ratio of the periplasmic β-lactam concentrations (17 °C/37°C) at steady-state hydrolysis by the extended spectrum β-lactamase. Experimental details are given in Materials and Methods. External β-lactam concentration was 100 μM. **A,** Experiments with the OprD-positive strains. **B,** experiments with the OprD-negative strains.

centrations were calculated to be 0.013 and 0.029 $\mu$M, respectively, by the PAO1 strain. The ratio of periplasmic cephalothin concentration at 17°C relative to that at 37°C appeared to be an average 0.43±0.09 (Fig. 1A). This value indicates that the diffusion rate of cephalothin at 17°C was about 40% of that at 37°C. This result implies that cephalothin mainly diffuses through the lipid domain of the outer membrane.

Next, we tested the impact of OprD-deficiency on the diffusion rate of imipenem at 17°C and 37°C. The periplasmic concentration of imipenem at steady-state in two OprD-negative strains at 17°C and 37°C dropped to 1.7 ~ 2.5 and 6.4 ~ 7.4 %, respectively, of the levels in the strain producing OprD. The ratio of the value at 17 °C relative to that at 37 °C was 0.22 and 0.43, respectively in the two strains, indicating clearly that the diffusion of imipenem in the OprD-negative strain takes place through the lipid phase (Fig. 1B).

### *Accumulation of Cell-Associated Tetracycline, Fluoroquinolone, and Chloramphenicol at 17 °C and 37 °C*

Another unanswered question is how porin-impermeable antibiotics cross the membrane. We measured the time course of accumulation of the antibiotics in the intact cell of TNP076, which lacks all the subunits of the Mex pump machinery, at 17°C and 37°C. Cell-associated tetracycline increased rapidly at 37°C and equilibrated at about 10 min of incubation. The accumulation at 17°C was slow and reached to about 35 % of the level of equilibrium even at 30 min (Fig. 2). The initial rate of tetracycline uptake at 17°C and 37°C appeared to be 0.09 $\mu$g and 1.43 $\mu$g per min per mg of protein, respectively, as 100 $\mu$g/ml of tetracycline was added externally. Thus, it became apparent that tetracycline accumulates at roughly 1/16 the velocity at 17°C compared with that at 37°C. This dramatic retardation of tetracyclin accumulation at low temperature suggests that the diffusion of tetracycline across the membrane(s) most likely takes place through the lipid domain of the membrane.

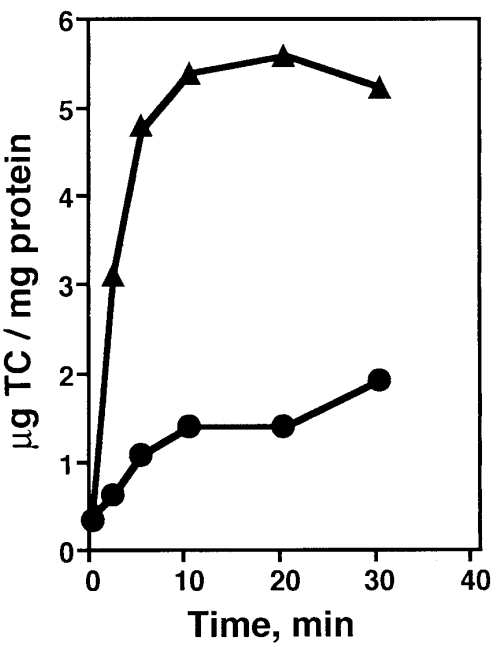


**FIG. 2.** Time course of determination of cell-associated tetracycline (TC). For experimental details, see the text. External tetracycline was 100 $\mu$g/ml. Several independent experiments confirmed the results. Symbols; ▲, 37°C; ●, 17°C.

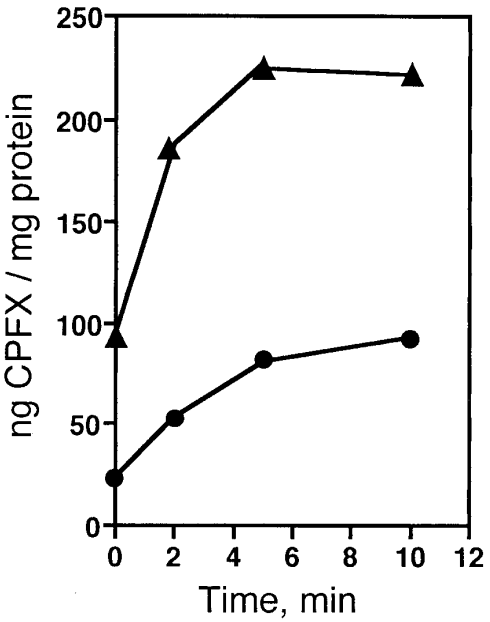


**FIG. 3.** Time course of determination of cell-associated ciprofloxacin (CPFX). For detail, see the text. External ciprofloxacin was 10 $\mu$g/ml. Data are representative of several independent assays. Symbols; ▲, 37°C; ●, 17°C.

In the next experiment, we determined the accumulation of the cell-associated fluoroquinolone antibiotic, ciprofloxacin at 37°C and 17°C. Ciprofloxacin rapidly accumulated at 37°C reaching an equilibrium at about 3 to 5 min, while the accumulation at 17°C was very slow (Fig. 3). A rough estimate of the initial rate of ciprofloxacin accumulation at 17°C and 37°C appeared to be 17.8 and 135 ng per min per mg of protein at 10 $\mu$g per ml of the external concentration. Thus, it is apparent that ciprofloxacin accumulates 7.5 times more slowly at 17 °C than at 37 °C.

Using a similar technique, we determined the accumulation of [$^{14}$C]-chloramphenicol at 10 $\mu$g per ml of the external concentration. The result depicted in Fig. 4 showed that the essential accumulation profile was similar to that of tetracycline and ciprofloxacin. The only minor difference observed was that the rates of accumulation at 17°C and 37°C were much closer than

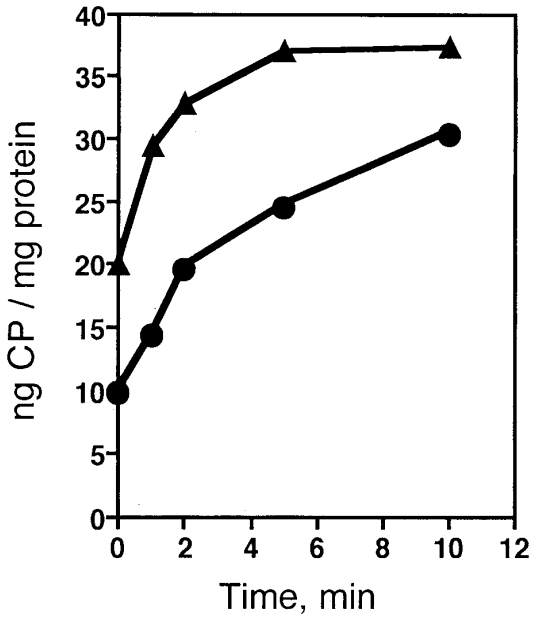


**FIG. 4.** Time course of determination of cell-associated chloramphenicol (CP). For details, see the text. External [$^{14}$C]-chloramphenicol concentration was 10 $\mu$g/ml. The result was confirmed by several independent assays.Symbols; ▲, 37°C; ●, 17°C.

in tetracycline and ciprofloxacin accumulation. The initial rate of chloramphenicol accumulation at 17°C was 2.5 times slower than that in 37°C. Thus, this also suggests that chloramphenicol preferentially diffuses through the lipid domain of the membrane.

## DISCUSSION

This paper reported that the diffusion rates of cephalosporin, tetracycline, chloramphenicol and fluoroquinolone were markedly retarded at 17°C compared with those at 37°C, whereas the diffusion rate of imipenem at 17°C was fully comparable with that at 37°C. In addition, the ratio (17°C/37°C) of the diffusion rate of imipenem in the OprD-negative strain dropped to an average of 0.32 from an average value of 1.03 in the OprD-positive strain. These results were interpreted to mean that cephalothin, tetracycline, chloramphenicol and fluoroquinolone diffuse through the outer membrane by dissolving in the lipid domain of the membrane. Imipenem crossed the outer membrane predominantly via the porin-channel in the OprD-positive strain, and through the lipid phase in the OprD-negative strain. These results were fully consistent with our previous results showing most antibiotics, excepting imipenem, were unable to diffuse through the *P. aeruginosa* porin channels (7).

In this study, we set the assay temperature to 17°C and 37°C. We reasoned this temperature setting as follows: (i) Lowering the assay temperature below 15°C causes massive leakage of $\beta$-lactamase as we reported earlier (13). (ii) It was reported that the liquid crystalline state of bacterial membrane lipid changes at 22 to 35°C (14). Accordingly, we set the lowest assay temperature to 17°C that might give a maximum temperature effect with a minimum leakage of $\beta$-lactamase. A temperature higher than 37°C caused fluctuation of the data making it difficult to obtain consistent results. Assays at temperatures below 37°C and above 17°C were successful, but the temperature effect on the diffusion rate was small at small temperature difference. Thus, after extensive preliminary experiments, assay temperatures were set to 17°C and 37°C.

We determined the effect of assay temperature on cell-associated fluoroquinolone, tetracycline, and chloramphenicol using the strain devoid of the antibiotic extrusion machinery, a MexA-MexB-OprM system. Use of this strain in the antibiotic accumulation experiments is particularly important, since the wild type *P. aeruginosa* expresses antibiotic extrusion machinery lowering the intracellular concentration of antibiotics (9,15). When we assayed cell-associated antibiotics, tetracycline and ciprofloxacin accumulated at 17°C at the rates of 1/16 and 1/8, respectively, compared with the rates at 37°C. Since the amount of antibiotic accumulated in these experiments may be the reflection of outer membrane and inner membrane permeability, it is difficult to conclude that the results reflected only outer membrane permeability. It was reported, however, that the lipid phase of the outer membrane is more rigid than the inner membrane (16). It is likely, therefore, that the rate limiting step of this antibiotic accumulation is the diffusion across the outer membrane.

In conclusion, cephalothin, fluoroquinolone antibiotics, tetracycline, and chloramphenicol assayed so far, diffused the outer membrane of *P. aeruginosa* mainly through the lipid domain of the bilayer and probably not through the porin channel. This explains why the outer membrane of this organism is tight barrier against many antibiotics.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Benz, R. (1988) *Ann. Rev. Microbiol.* **42,** 359–393.
2. Nikaido, H. (1992) *Molec. Microbiol.* **6,** 435–442.
3. Nakae, T. (1996) *Biochem. Biophys. Res. Commun.* **71,** 877–884.
4. Yoshimura, F., Zalman, L. S., and Nikaido, H. (1983) *J. Biol. Chem.* **258,** 2309–2314.

5. Yoneyama, H., Akatsuka, A., and Nakae, T. (1986) *Biochem. Biophys. Res. Commun.* **134,** 105–112.
6. Yoshihara, E., and Nakae, T. (1989) *J. Biol. Chem.* **264,** 6297–6301.
7. Yoneyama, H., Yamano, Y., and Nakae, T. (1995) *Biochem. Biophys. Res. Commun.* **213,** 88–95.
8. Quinn, J. P., Dudek, E. J., Divincenzo, C. A., Lucks, D. A., and Lerner, S. A. (1986) *J. Infect. Dis.* **154,** 289–294.
9. Yoneyama, H., Ocaktan, A., Tsuda, M., and Nakae, T. (1997) *Biochem. Biophys. Res. Commun.* **233,** 611–618.
10. Sambrook, J., Fritsch, E. F., and Maniatis, M. (1989) *in* Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
11. Zimmermann, W., and Rosselet, A. (1987) *Antimicrob. Agents Chemother.* **12,** 368–372.
12. Watanabe, M., Iyobe, S., Inoue, M., and Mitsuhashi, S. (1991) *Antimicrob. Agents Chemother.* **35,** 147–151.
13. Lei, Y., Satake, S., Ishii, J., and Nakae, T. (1991) *FEMS Microbiol. Lett.* **80,** 337–340.
14. Morrisett, J. D., Pownall, H. J., Plumlee, R. T., Smith, L. C., and Zehner, Z. E. (1975) *J. Biol. Chem.* **250,** 6969–6975.
15. Morshed, S. R. Md., Lei, Y., Yoneyama, H., and Nakae. T. (1995) *Biochem. Biophys. Res. Commun.* **210,** 356–362.
16. Schindler, M., Osborn, M. J., and Koppel, D. E. (1980) *Nature* **285,** 261–263.