Role of OmpD2 and chromosomal β-lactamase in carbapenem resistance in clinical isolates of *Pseudomonas aeruginosa*

S. Satake, H. Yoneyama and T. Nakae

Department of Cellular Information Sciences, Tokai University School of Medicine, Isehara 259-11, Japan

Imipenem-resistant clinical isolates of *Pseudomonas aeruginosa* were divided into two categories: (i) isolates that were moderately resistant to imipenem (MIC 6·25 mg/L) that produced trace amounts of protein D2 detected with immunoblotting using anti-protein D2 antibody, but not when stained with Coomassie blue and had inducible class 1 β-lactamase expression; (ii) isolates that were highly resistant to several β-lactams, including meropenem, with no protein D2 by staining or immunoblotting and had stably derepressed β-lactamase. Laboratory strains were isolated and analyzed: (i) mutants lacking protein D2, or (ii) lacking protein D2 and producing stably derepressed β-lactamase with carbapenem resistance similar to the clinical isolates. (iii) mutants producing undetectable β-lactamase which were four-fold more susceptible to imipenem than the mutant producing stably derepressed β-lactamase or the strain with inducible β-lactamase. These data suggests that β-lactamase and outer membrane permeability govern meropenem-resistance in *P. aeruginosa*.

Introduction

Nosocomial infections of *Pseudomonas aeruginosa* are a serious clinical problem, often due to the micro-organism's high intrinsic resistance to a number of antibiotics (Bryan, 1979). Although the reasons for this broad intrinsic resistance are not fully understood, it is generally assumed that there is poor antibiotic permeability across the outer membrane (Yoshimura & Nikaido, 1982), which is attributable to small-sized diffusion pores that exclude many antibiotics. A small β-lactam antibiotic, imipenem, overcame this difficulty by penetrating through the protein D2 pore (Satake, Yoshihara & Nakae, 1990; Trias & Nikaido, 1990). However, imipenem-resistant clinical isolates have been isolated after unsuccessful treatment of *P. aeruginosa* infections (Quinn *et al.*, 1986; Büscher *et al.*, 1987). It has been shown that most imipenem-resistant clinical isolates lacked the outer membrane protein D2 suggesting that decreased imipenem permeability could be the cause (Quinn *et al.*, 1986; Büscher *et al.*, 1987). It is possible, however, that both the diminished protein D2 production and derepressed β-lactamase synthesis are involved in resistance. In this study outer membrane proteins and β-lactamase from several imipenem-resistant clinical isolates were analyzed, and the data suggests that there are different causes of carbapenem resistance.

199

Materials and methods

Bacterial strains

P. aeruginosa TNP501 to TNP506 were from a recent collection from clinical sources at the University of Tokyo, School of Medicine. *P. aeruginosa* PA01 and four isogenic derivatives were also used. Strain TNP004 is a mutant selected for imipenem resistance *in vitro* and which produces an undetectable amount of protein D2. Strain TNP005 is a mutant selected for imipenem resistance from strain TNP001, a derivative of PAO1, producing constitutively derepressed β-lactamase and a normal level of protein D2. This strain lacks protein D2. Strain PAO4098 is a laboratory strain with defective *bla* gene(*met-9020, pro-9024, blaP-9208*).

Determination of susceptibility

The MIC of all the agents was determined by the agar dilution method using Mueller Hinton agar (Difco Laboratories, Detroit). Bacteria were grown overnight in Mueller Hinton broth (Difco) with shaking and were diluted to 10^6 cfu/mL. 5 μL of the cell suspension was inoculated onto agar plates containing two-fold serially diluted antibiotics. Growth of the cells was examined after overnight incubation at 37°C. Antibiotics were obtained from : imipenem (Banyu Pharmaceuticals, Tokyo); meropenem (Sumitomo Pharmaceuticals, Osaka); carbenicillin (Pfizer Taito Co., Ltd, Tokyo); piperacillin and cefoperazone (Toyama Chemicals, Tokyo); ceftazidime (Tanabe Pharmaceuticals, Osaka); cefsulodin (Takeda Chemicals, Osaka); aztreonam (Ezai Co., Ltd, Tokyo); ofloxacin (Daiichi Seiyaku Co., Ltd, Tokyo); gentamicin and cephalothin (Shionogi Pharmaceuticals, Osaka).

Preparation of outer membrane and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The outer membrane of *P. aeruginosa* was purified as described previously (Mizuno & Kageyama, 1978). 20 μg of outer membrane protein was subjected to SDS-PAGE (10% acrylamide) after heating at 95°C for 5 min in sample buffer (Laemmli, 1970). The gel was stained with Coomassie brilliant blue or immunoblotting (see below). Protein was quantified by the method of Lowry *et al.* (1951).

Western blot analysis

Adult female rabbits were immunized bi-weekly by a subcutaneous injection of 250 μg of protein D2 purified according to the procedure described previously (Yoshihara & Nakae, 1989; Yoshihara, Yoneyama & Nakae, 1991). The blood was collected ten days after the fourth immunization and the serum was separated. The anti-protein D2 antibody was purified with a sepharose-immobilized protein D2 column. Western blot analysis was carried out by the method described previously (Towbin, Staehelin & Gordon, 1979) with a few minor modifications (see below).

Cells were grown overnight in L-broth harvested by centrifugation, and suspended in a one-tenth volume of phosphate buffered saline (PBS), and then diluted ten-fold with 1·11% sodium dodecyl sulphate. The protein was quantified after boiling for 2 min at 100°C. Samples containing 30 μg protein were subjected to SDS-PAGE (10% acrylamide) and the protein bands were electrophoretically transferred to a polyvinylidene

difluoride (PVDF) membrane (Millipore, Bedford, MA) at 300 mA for 30 min. The PVDF sheet was immersed in PBS containing 3% bovine serum albumin for 30 min at 37°C, and then in PBS containing 2% of normal goat serum for 30 min at 23°C. The membrane was washed with PBS containing 0·5% Tween20 (T-PBS) and incubated with anti-protein D2 antibody in PBS containing 0·1% bovine serum albumin, 0·1% gelatin and 1 mM MgCl$_2$ for 60 min at 23°C. The membrane was incubated after washing with T-PBS containing 2% horseradish peroxidase-conjugated goat anti-rabbit IgG in T-PBS for 60 min at 23°C. The peroxidase was visualized by diaminobenzidine (0·25 g/L) in the presence of 0·006% H$_2$O$_2$. The sheet was then washed extensively with tap water.

β-Lactamase assays

Cells were grown overnight in L-broth containing 5 mM MgCl$_2$, diluted with a nine-fold volume of the same prewarmed medium and incubated at 37°C for 2 h with shaking at 180 rpm. 0·1 mg/L of imipenem was added and shaking continued at 180 rpm for 2 h at 37°C. Cells were harvested by centrifugation at 8000 g for 10 min at 30°C, suspended in an ice-cold 50 mM sodium phosphate buffer, pH 7·0, then fragmented by a French pressure cell at 500 kg/cm^2 using a 5 mL cell (Ohotake, Tokyo). Intact cells and debris were removed by centrifugation at 100,000 g for 1 h at 4°C. The enzyme activity in the supernatant was assayed spectrophotometrically at 262 nm at 30°C using 0·1 mM cephalothin as a substrate in 50 mM sodium phosphate buffer, pH 7·0.

Results

Antibiotic susceptibility

The imipenem-resistant clinical isolates could be classified into two categories on the basis of the antibiograms (Table I). One group of isolates, represented by strains TNP505 and TNP506 showed moderate MICs of imipenem, 6·25 mg/L (Table I) that was eight times higher than the MIC of PAO1 (Table II). Susceptibilities to other antibiotics were comparable with those of PAO1. Another group of isolates, represented by isolates TNP501, TNP502, TNP503, and TNP504 showed high MICs of

Table I. MICs of clinical isolates of *P. aeruginosa*

| Drug | MIC (mg/L) | | | | | |
	TNP501	TNP502	TNP503	TNP504	TNP505	TNP506
Imipenem	25	12·5	12·5	25	6·25	6·25
Meropenem	25	6·25	6·25	12·5	0·78	0·78
Carbenicillin	>100	>100	100	>100	25	50
Piperacillin	>100	>100	100	50	3·13	6·25
Cefoperazone	>100	>100	100	50	6·25	6·25
Ceftazidime	>100	50	25	12·5	3·13	3·13
Cefsulodin	100	50	25	12·5	1·56	3·13
Aztreonam	100	25	25	25	1·56	6·25
Ofloxacin	6·25	0·78	>100	1·56	3·13	0·39
Gentamicin	1·56	>100	1·56	50	3·13	3·13

Table II. MICs of *P. aeruginosa* PAO1 and its isogenic derivatives

| Drug | MICs (mg/L) | | | | |
	PAO1 wt	PAO4098 I⁻	TNP001 DR	TNP004 D⁻	TNP005 DR, D⁻
Imipenem	0·78	0·19	0·78	12·5	12·5
Meropenem	0·39	0·39	1·56	3·13	6·25
Carbenicillin	25	25	>100	50	>100
Piperacillin	3·13	3·13	>100	3·13	>100
Cefoperazone	3·13	3·13	>100	3·13	>100
Ceftazidime	1·56	1·56	100	1·56	100
Cefsulodin	1·56	1·56	50	1·56	50
Aztreonam	3·13	3·13	50	3·13	50
Ofloxacin	0·78	0·78	0·78	0·78	0·78
Gentamicin	3·13	3·13	3·13	3·13	3·13

wt, Wild type; I⁻, non-inducible; low level β-lactamase; DR, derepressed β-lactamase; D⁻, protein D2-defective.

imipenem and meropenem, exhibiting values of 6·25–25 mg/L (Table I) that were 16–64 times the value of the MIC of PAO1. MICs of other β-lactam antibiotics were also high (Table I).

Imipenem-resistant mutants were selected from PAO1 *in vitro* with 6·25 mg/L imipenem. All 30 selected mutants, e.g. TNP004, showed an eight- to 16-fold increase in the MIC of imipenem (Table II), and no change to the susceptibility to other antimicrobial agents. This profile was similar to that seen for the clinical isolates TNP505 and TNP506, except that the MIC of meropenem was eight times higher than that of PAO1 (Table II). PAO4098 produced undetectable β-lactamase and was four times more susceptible to imipenem than the β-lactamase inducible strain, PAO1 (Table II). There was no difference in the susceptibility to other β-lactams.

The β-lactamase constitutive strain, TNP001, was selected from PAO1 at 16 times MIC of ceftazidime (25 mg/L), and was 64-fold less susceptible to ceftazidime than PAO1. Interestingly, TNP001 was four-fold less susceptible to imipenem and meropenem than PAO4098. The imipenem-resistant mutants selected from TNP001 (e.g. TNP005) had a 16-fold increase in the MIC of imipenem and meropenem compared to PAO1, and the MICs of all other β-lactam antibiotics were also significantly higher than those for PAO1 and TNP004. However, susceptibility to ofloxacin and gentamicin were unchanged. This profile was similar to that of clinical isolates TNP501, TNP502, TNP503 and TNP504. Thus, we were able to simulate the resistant isolates by selecting resistant mutants *in vitro*.

Outer membrane protein profiles

Figure 1 shows the outer membrane protein profiles of clinical isolates, PAO1 and PAO1 mutants. All the imipenem-resistant isolates, regardless of their sources, lacked protein D2 (Figure 1). All other protein bands were comparable with those of PAO1 excepting isolate TNP506 which showed a new protein band with an apparent M_r of 40,000 and a decreased density of the protein G band.

When the gel was visualized by immunoblotting staining, only in PAO1 was the band corresponding to protein D2 densely stained. The lanes for strains TNP501, TNP502,
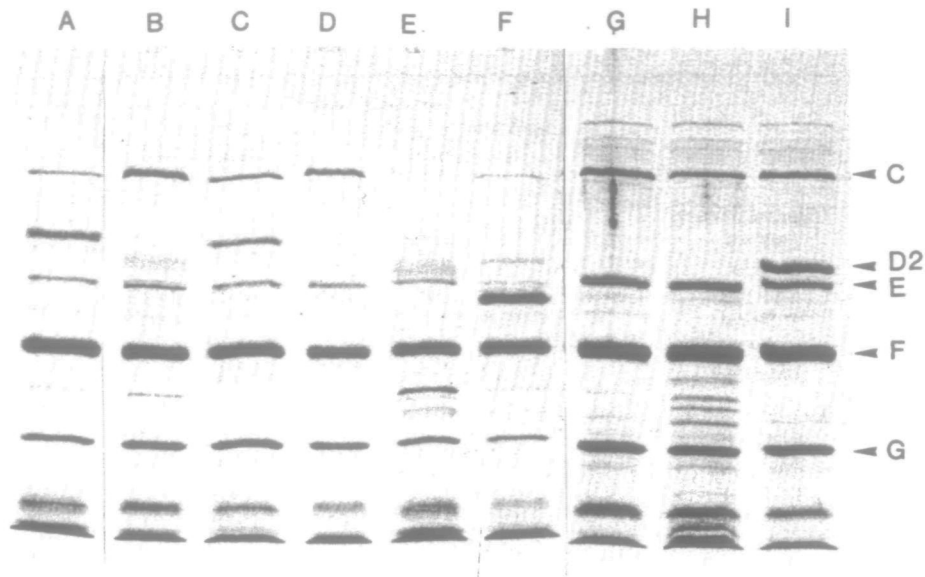
Figure 1. Outer membrane protein profiles of nine *P. aeruginosa*. Nomenclature of the outer membrane proteins is that of Mizuno & Kageyama (1978). Lane A, TNP501; lane B, TNP502; lane C, TNP503; lane D, TNP504; lane E, TNP505; lane F, TNP506; lane G, TNP005; lane H, TNP004; lane I, PAO1.
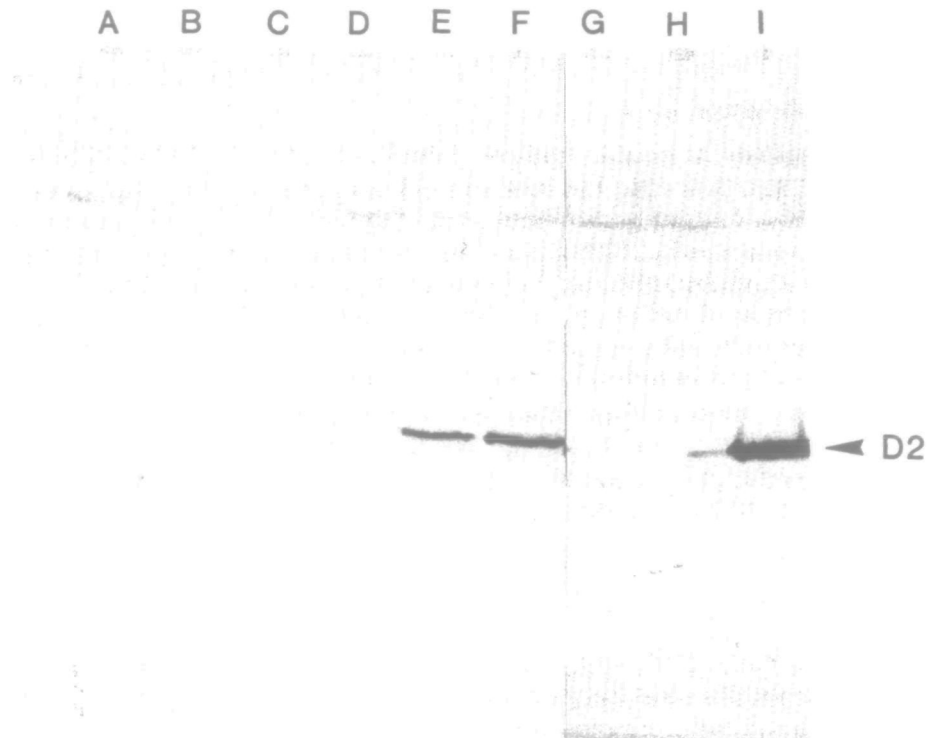

Figure 2. Western blot analysis of the outer membrane protein D2. Lane A, TNP501; lane B, TNP502; lane C, TNP503; lane D, TNP504; lane E, TNP505; lane F, TNP506; lane G, TNP005; lane H, TNP004; lane I, PAO1.

Table III. β-Lactamase activities of clinical isolates and isogenic laboratory strains of *P. aeruginosa*

Strain	Specific activity[a] unit/mg of protein in the presence of:	
	no inducer	imipenem
Clinical isolates		
TNP501	7·147 ± 0·880	10·350 ± 1·178
TNP502	2·633 ± 1·200	6·947 ± 2·342
TNP503	2·730 ± 0·471	6·077 ± 3·111
TNP504	1·125 ± 0·201	5·320 ± 0·629
TNP505	0·041 ± 0·040	0·756 ± 0·124
TNP506	0·021 ± 0·014	0·746 ± 0·111
Isogenic strains		
PAO1	0·008 ± 0·002	1·500 ± 0·695
PAO4098	0·007 ± 0·003	0·004 ± 0·002
TNP001	6·750[b]	6·520[b]
TNP004	0·008[b]	0·390[b]
TNP005	4·940[b]	7·730[b]

[a]Specific activity was expressed mean ± S.D., unless otherwise noted.
[b]Average of two independent assays.

TNP503, TNP504, TNP004 and TNP005 showed no detectable protein, and strains TNP505 and TNP506 showed faint protein bands corresponding to protein D2

Analysis of β-lactamase activity

β-Lactamase activity in isolates TNP501, TNP502, TNP503, TNP504, TNP001 and TNP005 were 855, 314, 326, 134, 844 and 618 times respectively, higher than the activity of PAO1, suggesting that these strains produced β-lactamase constitutively. β-Lactamase production in TNP505 and TNP506 was only slightly higher than that of PAO1. TNP004 and PAO4098 had comparable β-lactamase activity with PAO1.

The activity of the β-lactamases were examined from cells grown in the presence of 0·1 mg/L imipenem. Imipenem was a potent inducer, elevating the enzyme activity to 179 times that of uninduced PAO1, TNP505 and TNP506 had 18 and 36 times higher enzyme activities when induced (Table III), and TNP004 showed 49 times higher activity upon induction. The isolates and mutants with high enzyme activity without inducer had negligible changes in enzyme activity in the presence of imipenem (Table III). PAO4098 had little detectable β-lactamase activity in the presence of inducer.

Discussion

The natural resistance of *P. aeruginosa* to many antibiotics may be due to low outer membrane permeability and the production of antibiotic modifying enzymes (Bryan, 1979). Low outer membrane permeability is most likely due to the presence of a limited number of small-sized porin pores (Yoshihara & Nakae, 1989). To overcome this diffusion barrier, the development of small molecule antibiotics has been long awaited. Imipenem satisfied this requirement and has been powerful in *P. aeruginosa* therapy.

Recently, the emergence of imipenem-resistant clinical isolates, remaining susceptible to most other antibiotics has been reported (Quinn *et al.*, 1986; Büscher *et al.*, 1987). Analysis of such imipenem-resistant isolates showed markedly diminished outer membrane protein D2 suggesting that the imipenem resistance is attributed to a permeability defect. The reason that the protein D2 defective mutants were still susceptible to other antibiotics is probably that the small-sized porin pores play only a small role in the diffusion of antibiotics other than carbapenems. It was suggested that protein D2 facilitate the diffusion of imipenem specifically (Trias & Nikaido, 1990). Recently, it was observed that in imipenem-resistant *P. aeruginosa* isolated clinical laboratories in Japan expressed high level resistance to other β-lactam antibiotics (Goto, Goto & Shimada, 1989). Analysis of the outer membrane proteins of the imipenem-resistant clinical isolates showed that all the mutants lacked protein D2 confirming previous reports (Quinn *et al.*, 1986; Büscher *et al.*, 1987). Using immuno-blotting with anti-protein D2 serum it was shown that isolates TNP505 and TNP506 produced trace amounts of protein D2 and were moderately resistant to imipenem (MIC = 6·25 mg/L), but relatively susceptible to meropenem. Since this was not the case in mutant TNP004 which lacked protein D2, the meropenem activity for TNP505 and TNP506 is likely to be attributed to the presence of a trace amount of protein D2.

It was reported that carbenicillin-resistant *P. aeruginosa* are resistant to meropenem and other antibiotics, but not to imipenem (Livermore & Yang, 1987) suggesting that meropenem diffuses through the outer membrane via two routes. One is the route through which meropenem, carbenicillin and other unrelated antibiotics such as norfloxacin diffuse (Livermore, 1984); TNP501 may have a mutation with this pathway. The alternative pathway is the route through which only carbapenems can pass (Trias & Nikaido, 1990). Such a route in *P. aeruginosa* may be the protein-D2 pore.

Clinical isolates, TNP501, TNP502, TNP503 and TNP504 showed elevated MICs of imipenem, meropenem and most other β-lactam antibiotics. These mutants lack protein D2, and produce class 1 β-lactamase constitutively (Table III). The data suggests that high imipenem and meropenem resistance (MIC 12·5 to 25 mg/L) may be achieved by decreasing carbapenem diffusion (lack of protein D2) and elevated hydrolysis of β-lactams by the constitutively derepressed β-lactamase. The role of protein D2 in carbapenem susceptibility is shown by comparison of the MIC of carbapenems in mutants TNP001 and TNP005. The protein D2 defective strain, TNP005, showed 16 and four times higher MIC of imipenem and meropenem, respectively, than TNP001. The role of β-lactamase is shown by comparison of the MICs of carbapenems in strain PAO4098 and TNP001 producing undetectable and constitutively elevated β-lactamase, respectively. The β-lactamase constitutive mutant was four times less susceptible to carbapenems than the mutant with no β-lactamase. The role of induced β-lactamase was analyzed using strains PAO4098 and PAO1 with uninducible low and inducible β-lactamase respectively. The MIC of imipenem for PAO1 was four times higher than PAO4098, whereas the MIC of meropenem for these strains were comparable. The difference in the MIC values of imipenem and meropenem suggest that meropenem is a poor inducer (Yang & Livermore, 1989; S. Satake & T. Nakae, unpublished data). This study shows that clinical isolates of imipenem resistant *P. aeruginosa* could be sub-divided into two categories (i) strains lacking protein D2; and (ii) strains lacking protein D2 and producing stably derepressed class 1 β-lcatamase. We confirmed the appearance of the protein D2-defective mutant by selecting for imipenem-resistance

and the protein D2-defective plus β-lactamase constitutive mutant by selecting firstly with ceftazidime and then by imipenem. It is likely that the highly carbapenem-resistant clinical isolates were selected by the use of carbapenem and other β-lactam antibiotics during therapy for *P. aeruginosa* infection.

References

Bryan, L. E. (1979). Resistance to antimicrobial agents: The general nature of the problem and the basis of resistance. In *Pseudomonas aeruginosa. Clinical Manifestations of Infection and Current Therapy.* (Doggett, R. G., Ed.), pp. 219–70. Academic Press, London.

Büscher, K. H., Cullmann, W., Dick, W., Wendt, S. & Opferkuch, W. (1987). Imipenem resistance in *Pseudomonas aeruginosa* is due to diminished expression of outer membrane proteins. *Journal of Infectious Diseases* **156**, 681–4.

Goto, M., Goto, H. & Shimada, K. (1989). Frequency of isolation of *Pseudomonas aeruginosa* in 19 domestic hospitals and their drug susceptibility. *Annual Meeting of Japanese Society of Chemotherapy.* Abstract 232, p. 180.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.

Livermore, D. M. (1984). Penicillin-binding proteins, porins and outer-membrane permeability of carbenicillin-resistant and -susceptible strains of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology* **18**, 261–70.

Livermore, D. M. & Yang, Y. J. (1987). β-Lactamase lability and inducer power of newer β-lactam antibiotics in relation to their activity against β-lactamase-inducibility mutants of *Pseudomonas aeruginosa*. *Journal of Infectious Diseases* **155**, 775–82.

Livermore, D. M. & Yang, Y. (1989). Comparative activity of meropenem against *Pseudomonas aeruginosa* strains with well-characterized resistance mechanisms. *Journal of Antimicrobial Chemotherapy* **24**, Suppl. A, 149–59.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–75.

Mizuno, T. & Kageyama, M. (1978). Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. *Journal of Biochemistry* **84**, 179–91.

Quinn, J. P., Dudek, E. J., DiVincenzo, C. A., Lucks, D. A. & Lerner, S. A. (1986). Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. *Journal of Infectious Diseases* **154**, 289–94.

Satake, S., Yoshihara, E. & Nakae, T. (1990). Diffusion of β-lactam antibiotics through liposome membranes reconstituted from purified porins of the outer membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**, 685–90.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4.

Trias, J. & Nikaido, H. (1990). Outer membrane protein D2 catalyzed diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**, 52–7.

Yang, Y. & Livermore, D. M. (1989). Interactions of meropenem with class I chromosomal β-lactamases. *Journal of Antimicrobial Chemotherapy* **24**, Suppl. A, 207–17.

Yoshihara, E. & Nakae, T. (1989). Identification of porins in the outer membrane of

Pseudomonas aeruginosa that form small diffusion. *Journal of Biological Chemistry* **264**, 6297–301.

Yoshihara, E., Yoneyama, H. & Nakae, T. (1991). *In vitro* assembly of the functional porin trimer from dissociated monomers in *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* **266**, 952–7.

Yoshimura, F. & Nikaido, H. (1982). Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *Journal of Bacteriology* **152**, 636–42.