Leading articles

Intrinsic antibiotic resistance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, an opportunistic pathogen, is one of the major causes of life-threatening bacterial infections in western society. It is particularly troublesome in those patients who are already debilitated by severe burns, cancer, cystic fibrosis, leukaemia, diabetes mellitus, or major surgery including transplantation. Owing to the high intrinsic resistance of *P. aeruginosa* to almost all commonly used antibiotics, infections caused by this pathogen, once established, are often fatal (Flick & Cluff, 1976). In contrast to many other bacteria, plasmid-mediated antibiotic resistance is relatively uncommon (Bryan, 1979). This led a number of researchers (Nordstrom and Sykes, 1974; Brown, 1975; Bryan, 1979) to propose that the cell wall of *P. aeruginosa* might be a barrier to antibiotic diffusion although, until recently, direct evidence to support this conclusion was lacking.

It is now well established that the predominant reason for this high intrinsic resistance to hydrophilic antibiotics, such as β-lactams, is the low rate of permeation of these antibiotics across the outer membrane (Angus *et al.*, 1982; Yoshimura & Nikaido, 1982; Nicas & Hancock, 1983). The rate of permeation of β-lactam compounds across the outer membrane of *P. aeruginosa* is 12–100 fold lower than the rate of permeation of the same compounds across the outer membranes of *Escherichia coli*. The relationship between low outer membrane permeability and antibiotic resistance was confirmed by examination of a mutant strain of *P. aeruginosa* (Z61) which is highly susceptible to all 27 antibiotics studied (Zimmerman, 1979; Angus *et al.* 1982). The mutant has a six fold increase in outer membrane permeability to the chromogenic β-lactam nitrocefin, compared with its present strain K799 which has normal antibiotic resistance (Angus *et al.*, 1982).

In other Gram-negative bacteria, it has been demonstrated that 'porin' proteins, which form water-filled channels across the outer membrane, are responsible for the uptake, into the cell periplasm, of hydrophilic antibiotics, such as β-lactams (Nikaido & Vaara, 1985). A mutant strain of *P. aeruginosa* lacking a major outer membrane protein, designated protein F, was shown to have a seven fold decrease in outer membrane permeability compared to its parent strain (Nicas & Hancock, 1983). This suggests that protein F is the major protein responsible for the (albeit poor) outer membrane permeation pathway of *P. aeruginosa*. In addition, purified protein F has been demonstrated to form water-filled channels in model membranes by three different methods (Hancock, Decad & Nikaido, 1979; Benz & Hancock, 1981; Yoshimura *et al.*, 1983).

There has been some controversy recently over the actual size of the water-filled channels across the outer membrane of *P. aeruginosa*. Our original data (Hancock & Nikaido, 1978; Hancock *et al.*, 1979), based on the liposome exclusion assay, suggested that protein F and protein F-containing outer membrane fragments formed very large water-filled channels with an exclusion limit for saccharides of approximately 3000 daltons compared to the 600 dalton exclusion limit of *Esch. coli* pore-forming (porin) proteins. This paradox was resolved when data were presented (Benz & Hancock, 1981; Yoshimura *et al.* 1983) indicating that only a small percentage, perhaps as few as 0·4% of the approximately 200 000 protein F molecules per cell (Nicas & Hancock, 1983), formed these large channels. Therefore, while the *P. aeruginosa* outer membrane has a large exclusion limit due to these large channels, the small total area of channels available for diffusion of compounds as large as antibiotics results in low outer membrane permeability. Interestingly, we have recently obtained evidence suggesting that the other 99·6% or so of protein F channels are small and should be almost impenetrable by β-lactams (Woodruff *et al.* 1986), in contrast to our previous proposal that these other channels were closed (Nicas & Hancock, 1983).

Further evidence for the proposed large exclusion limit was provided by Miller & Becker (1978), who demonstrated that *P. aeruginosa* grew as well on pentamethionine as it did on the free amino acid methionine, whereas an *Esch. coli* methionine auxotroph grew well on trimeth-

ionine and methionine-containing tetrapeptides but did not grow on pentamethionine (Becker & Naider, 1974). Furthermore, large side chains attached to β-lactams often reduce the activity of these β-lactams against enteric organisms, such as *Esch. coli*, but not against *P. aeruginosa*. Two recent papers have disputed the concept of an overall large exclusion limit due to a small number of large pores (Caulcott, Brown & Gouda, 1984; Yoneyama, Akatsuka & Nakal, 1986). However, I believe that these papers have flaws in experimental design which detract from the conclusions made in them. For example, the paper of Caulcott *et al.* (1984) utilizes a series of centrifugation procedures on cells loaded with radiolabelled saccharides of different sizes. The results are analysed by examining the relative rates of leakage of these saccharides over a long period. The leakage of saccharides is expressed relative to that of sucrose. The conclusion that the *P. aeruginosa* exclusion limit is small is based on the assumption that larger substrates which can permeate across the outer membrane should permeate at the same rate as sucrose, an assumption that is clearly incorrect given the theoretical considerations of Renkin (1954), who demonstrated that friction of a trisaccharide passing through a pore of limited dimensions must be very much greater than friction on a disaccharide passing through the same channel. In addition, outer membrane permeability measurements cannot be made on cells that have been washed after loading with radiolabelled compounds, since the time required for half equilibration of solutes across the outer membrane is in the order of a few seconds. To estimate the exclusion limit, the paper of Yoneyama *et al.* (1986) utilizes plasmolysis experiments, based on the ability of large concentrations of saccharides to pass across the outer but not the inner membrane, causing water to leave the cytoplasm and consequent collapse of the inner membrane. However both plasmolysis and outer membrane collapse reflect the relative rate of trans-outer membrane permeability to the sugar in question compared with the rate of water movement across the cytoplasmic membrane. Thus, for *P. aeruginosa* these experiments can be interpreted by proposing that the rate of penetration, even of sucrose, across the outer membrane is too slow to prevent the collapse of the outer membrane due to water leakage across the cytoplasmic and, subsequently, the outer membrane. It should be noted that the above papers differ in their estimates of the size of sugar that can permeate the *P. aeruginosa* outer membrane: disaccharides in the case of Caulcott *et al.* (1984) and monosaccharides in the case of Yoneyama *et al.* 1986.

One important consideration is whether low outer membrane permeability alone is sufficient to account for the level of resistance to all antibiotics observed in *P. aeruginosa*. Despite the low rate of diffusion across the *P. aeruginosa* outer membrane one can calculate that the β-lactam nitrocefin, at an external concentration of 5 mg/l (given the measured outer membrane permeability coefficient C of $1 \cdot 3 \times 10^{-3}$ min^{-1} mg cells^{-1} ml) would equilibrate across the outer membrane in 21 sec. Thus low outer membrane permeability will slow down the rate of uptake into the periplasm (to 116 molecules of β-lactam taken up/cell/sec in the above example) but will not prevent uptake. A secondary defence mechanism such as the presence of a β-lactamase is required to prevent the build-up of β-lactams in the periplasm to the same concentration as that in the external medium. This is emphasized by the demonstration of Vu & Nikaido (1985) that strains of β-lactamase-derepressed *Enterobacter cloacae* are resistant to third generation cephalosporins as a result of low outer membrane permeability combined with very slow hydrolysis in the periplasm. These observations have been recently confirmed for β-lactamase derepressed isolates of *P. aeruginosa* (Livermore, 1985; Bayer, A. S., Parr, T. R. Jr., Chan, L. and Hancock, R. E. W. unpublished, observations). Therefore it appears there are at least two readily demonstrable factors which interact to give rise to resistance to β-lactams in *P. aeruginosa* low outer membrane permeability and hydrolysis of incoming β-lactams by periplasmic β-lactamase.

Many non-β-lactam antibiotics probably also utilize the porin pathway of *P. aeruginosa*. However, polycationic antibiotics, such as aminoglycosides and polymyxins, traverse the outer membrane by a different route (Hancock, 1984). These antibiotics interact with the outer membrane at sites where divalent cations form cross-bridges between adjacent lipopolysaccharide molecules (Peterson, Hancock & McGroarty, 1985; Moore, Bates & Hancock, 1986). The consequent displacement of these divalent cations by the bulkier polycationic antibiotics (Moore *et al.*, 1986) results in an increase in outer membrane permeability (Hancock, Raffle & Nicas, 1981; Loh, Grant & Hancock, 1984) which has been proposed to result in increased uptake of the polycationic antibiotic itself (Hancock, 1984). Since these polycations are promoting their own uptake across the outer membrane we have called this phenomenon 'self-promoted' uptake. The presence of this alternative pathway for aminoglycosides may explain the relative efficacy of such compounds

against *P. aeruginosa*. Furthermore, since it has been demonstrated, that aminoglycosides will disrupt the outer membrane thereby increasing its permeability towards the β-lactam nitrocefin (Hancock *et al.*, 1981), this presents a plausible explanation of the known synergy between β-lactams and aminoglycosides against *P. aeruginosa* (Sykes & Morris, 1975).

Since low outer membrane permeability presents a considerable problem in the therapy of *P. aeruginosa* infections and since activation of the 'self promoted' uptake pathway results in an increase in outer membrane permeability (Hancock *et al.*, 1981; Loh *et al.*, 1984), a new approach to *P. aeruginosa* therapy can be proposed. Presentation of an activator of the self-promoted pathway together with an antibiotic may result in increased efficacy of this antibiotic against *P. aeruginosa* infections. With this in mind, we (Hancock & Wong, 1984) instituted a screening systems for such activator compounds which we have termed 'permeabilizers'. These compounds fall into four basic classes: polycations, divalent cation chelators, monovalent organic cations and, possibly, reducing agents. A number of these compounds including polymyxin B (Sykes & Morris, 1975), polymyxin B nonapeptide (Vaara & Vaara, 1983), aminoglycosides (Sykes & Morris, 1975), ascorbate (Rawal, McKay & Blackhall, 1974) and EDTA (Wilson, 1970) have demonstrated effective synergy with antibiotics.

While these studies have become quite sophisticated, I feel that they have only touched upon the problems involved in treating *P. aeruginosa* infections. The considerable difficulties that remain include the development of unstable resistance to antibiotics (adaptation to resistance that reverts upon antibiotic removal) (e.g. Gerber & Craig, 1982), the common isolation of low level antibiotic-resistant mutants with subtle surface and permeability alterations (Bryan, O'Hara & Wong, 1984; Godfrey, Hatledid & Bryan, 1984; Godfrey, 1984) and the poor relationship between laboratory-derived MICs and therapeutic efficacy (Davis, 1974; Flick & Cluff, 1976).

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References

Angus, B. L., Carey, A. M., Caron, D. A., Kropinski, A. M. B. & Hancock, R. E. W. (1982). Outer membrane permeability in *Pseudomonas aeruginosa*: Comparison of a wild type with an antibiotic super-susceptible mutant. *Antimicrobial Agents and Chemotherapy* 21, 229–309.

Becker, J. M. & Naider, F. (1974). Stereospecificity of tripeptide utilization in a methionine auxotroph of *Escherichia coli* K-12. *Journal of Bacteriology* 120, 191–6.

Benz, R. & Hancock, R. E. W. (1981). Properties of the large ion permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. *Biochimica et Biophysica Acta* 646, 298–308.

Brown, M. R. W. (1975). The role of the cell envelope in resistance. In *Resistance of Pseudomonas aeruginosa* (Brown, M. R. W., Ed.), pp. 71–107. John Wiley, Chichester.

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. 219–270. Academic Press, New York.

Bryan, L. E., O'Hara, K. & Wong, S. (1984). Lipopolysaccharide changes in impermeability-type aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 26, 250–5.

Caulcott, C. A., Brown, M. R. W. & Gouda, I. (1984). Evidence for small pores in the outer membrane of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* 21, 119–23.

Davis, S. D. (1974). Dissociation between results for *in vitro* and *in vivo* antibiotic susceptibility tests for some strains of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 5, 281–8.

Flick, M. R. & Cluff, L. E. (1976). *Pseudomonas* bacteremia, a review of 108 cases. *American Journal of Medicine* 60, 501–8.

Gerber, A. U. & Craig, W. A. (1982). Aminoglycoside-selected subpopulations of *Pseudomonas aeruginosa*. Characterization and virulence in normal and leukopenic mice. *Journal of Laboratory and Clinical Medicine* 100, 671–81.

Godfrey, A. J., Hatledid, L. & Bryan, L. E. (1984). Correlation between lipopolysaccharide structure and permeability resistance in β-lactam-resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 26, 181–6.

Hancock, R. E. W. (1984). Alterations in outer membrane permeability. *Annual Review of Microbiology* 38, 237–64.

Hancock, R. E. W., Decad, G. M. & Nikaido, H. (1979). Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PA01. *Biochimica et Biophysica Acta* 554, 323–9.

Hancock, R. E. W. & Nikaido, H. (1978). Outer membrane of Gram negative bacteria. XIX Isolation from *Pseudomonas aeruginosa* and use in reconstitution and definition of the permeability barrier. *Journal of Bacteriology* 136, 381–90.

Hancock, R. E. W., Raffle, V. J. & Nicas, T. I. (1981). Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 19, 771–83.

Hancock, R. E. W. & Wong, P. G. W. (1984). Compounds which increase the permeability of the Pseudomonas aeruginosa outer membrane. Antimicrobial Agents and Chemotherapy 26, 48–52.

Livermore, D. M. (1985). Do β-lactamases 'trap' cephalosporins? Journal of Antimicrobial Chemotherapy 15, 511–4.

Loh, B., Grant, C. & Hancock, R. E. W. (1984). Use of the fluorescent probe 1-N-phenylnapthylamine to study the interaction of aminoglycoside antibiotics with the outer membrane of Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy 26, 546–51.

Miller, R. V. & Becker, J. M. (1978). Peptide utilization in Pseudomonas aeruginosa: evidence for a membrane associated peptidase. Journal of Bacteriology 133, 165–71.

Moore, R. A., Bates, N. C. & Hancock, R. E. W. (1986). Interaction of polycationic antibiotics with Pseudomonas aeruginosa lipopolysaccharide and lipid A studied using Dansyl polymyxin. Antimicrobial Agents and Chemotherapy 29, 496–500.

Nicas, T. I. & Hancock, R. E. W. (1983). Outer membrane permeability in Pseudomonas aeruginosa: Isolation of a porin protein F-deficient mutant. Journal of Bacteriology 153, 281–5.

Nikaido, M. & Vaara, M. (1985). Molecular basis of the permeability of the bacterial outer membrane. Microbiology Reviews 49, 1–32.

Nordstrom, K. & Sykes, R. B. (1974). Induction kinetics of beta-lactamase biosynthesis in Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy 6, 734–40.

Peterson, A. A., Hancock, R. E. W. & McGroarty, E. J. (1985). Binding of polycationic antibiotics to lipopolysaccharides of Pseudomonas aeruginosa. Journal of Bacteriology 164, 1257–61.

Rawal, B. D., McKay, G. & Blackhall, M. I. (1974). Inhibition of Pseudomonas aeruginosa by ascorbic acid acting singly and in combination with antimicrobials: in vitro and in vivo studies. Medical Journal of Australia 1, 169–74.

Renkin, E. M. (1954). Filtration, diffusion and molecular sieving through porous cellulose membranes. Journal of General Physiology 38, 225–243.

Sykes, R. B. & Morris, A. (1975). Resistance of Pseudomonas aeruginosa to antimicrobial drugs. Progress in Medicinal Chemistry 12, 333–93.

Vaara, M. & Vaara, T. (1983). Polycations sensitize enteric bacteria to antibiotics. Antimicrobial Agents and Chemotherapy 24, 107–13.

Vu, M. & Nikaido, M. (1985). Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive Enterobacter cloacae strain to expanded-spectrum β-lactams. Antimicrobial Agents and Chemotherapy 27, 393–8.

Wilson, L. A. (1970). Chelation in experimental Pseudomonas aeruginosa keratitis. British Journal of Opthalmology 54, 587–93.

Woodruff, W. A., Parr, T. R., Jr., Hancock, R. E. W., Hanne, L. Nicas, T. I. & Iglewski, B. (1986). Expression in Escherichia coli and function of porin protein F of Pseudomonas aeruginosa. Journal of Bacteriology 16, 473–9.

Yoneyama, H., Akatsuka, A. & Nakae, T. (1986). The outer membrane of Pseudomonas aeruginosa is a barrier against the penetration of disaccharides. Biochemical and Biophysical Research Communications 134, 106–12.

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

Yoshimura, F., Zalman, L. S. & Nikaido, H. (1983). Purification and properties of Pseudomonas aeruginosa porin. Journal of Biological Chemistry 258, 2308–14.

Zimmermann, W. (1979). Penetration through the gram-negative cell wall: a co-determinant of the efficacy of beta-lectam antibiotics. International Journal of Clinical Pharmacology and Biopharmacology 17, 131–4.

The prevention of wound infection after coronary artery bypass surgery

Sternal wound infection, one of the many possible complications of open heart surgery, has a sinister reputation and may progress to mediastinitis, osteomyelitis and bacteraemia; it has a high mortality (7–45%) and the survivors often require extensive wound debridement (Sarr, Gott & Townsend, 1984). Risk factors for sternal sepsis have recently been reviewed by Sarr et al. (1984).

Although both coronary artery bypass graft (CABG) and valve replacement operations are conventionally "clean" and require cardiopulmonary bypass (CPB), in CABG no intravascular prosthesis is involved but instead saphenous veins are harvested from the upper thigh. Two recent reports emphasized the differences between the two operations and related them to post-operative infection (Wells, Newsom & Rowlands, 1983; Farrington et al., 1985a). Both found sternal wound sepsis to be common after CABG (about 8%) and to be caused by Staphylococcus aureus or endogenous, antibiotic-sensitive coliforms; coliforms were also often isolated from leg incision infections. Sternal infection after valve replacement was less common (about 2%) and predominantly staphylococcal. Available evidence suggests peri-operative implantation of pathogens; Kluge et al. (1974) found extensive contamination of chest wounds in theatre with skin commensals, but coliforms and S. aureus were occasionally also isolated. During CPB the patient may be exposed to wound contaminants via the blood stream since blood (with theatre air and other contents of the wound) is aspirated from around the heart and returned to the circulation. Wells et al. (1983) suggested that bowel flora was