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Interplay of Impermeability and Chromosomal β-Lactamase Activity in Imipenem-Resistant *Pseudomonas aeruginosa*

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Mutational loss of the D2 porin causes imipenem resistance in *Pseudomonas aeruginosa*. It was found that this mechanism could function only when the chromosomal β-lactamase was expressed. Mutants lacking both the β-lactamase and the D2 porin were almost as susceptible as those that lacked the β-lactamase but retained the porin. Thus, imipenem resistance reflected an interplay of the enzyme and impermeability, not either factor alone. These findings suggest that the activity of a carbapenem more β-lactamase stable than imipenem should be less affected by the porin loss. Meropenem approached this behavior.

Most *Pseudomonas aeruginosa* strains are inducible for a chromosomal β-lactamase placed in group 1 of Bush's classification (5), class C of Ambler's classification (8), and class I of Richmond and Sykes' scheme (15). Imipenem induces this enzyme strongly and is hydrolyzed slowly by it (turnover number [k_{cat}] = 1/min, compared with 17,000/min for cephaloridine, 4,500/min for benzylpenicillin, and 15/min for cefotaxime) (10). Thus, the MICs of imipenem for β-lactamase-inducible strains and those for their derepressed mutants are equal (1 to 2 μg/ml), whereas β-lactamase-deficient mutants are more susceptible (MICs, 0.12 to 0.25 μg/ml) (10). Most antipseudomonal cephalosporins and penicillins are hydrolyzed more rapidly than imipenem by the β-lactamase but do not induce its synthesis. Consequently, their MICs for β-lactamase-inducible strains are as low as those for basal mutants, whereas derepressed mutants are more resistant (6, 10). An important consequence of these observations is that the penicillins and cephalosporins tend to select derepressed mutants from inducible populations, whereas imipenem does not (3, 10). Nevertheless, imipenem resistance can arise readily in *P. aeruginosa* via mutational loss of the D2 porin (4, 13). This protein forms outer membrane pores that are permeable to carbapenems but not to penicillins and cephalosporins (16). It remains uncertain, however, whether the porin loss alone causes imipenem resistance or whether the feeble activity of the β-lactamase is also necessary. In order to investigate this aspect, *P. aeruginosa* mutants deficient for both the D2 porin and the β-lactamase were derived and examined.

Derivation and characterization of mutants. *P. aeruginosa* M1405β-con and M2297β-con were β-lactamase-derepressed clinical isolates; M2297P was the β-lactamase-inducible parent strain of M2297β-con; and M1405β-def and M2297β-def were β-lactamase basal mutants of M1405β-con and M2297β-con, respectively. All of these organisms have been described previously (10). None had any secondary β-lactamase in addition to the chromosomal type. M1405β-con D2$^-$ and M2297β-con D2$^-$ were spontaneous mutants of M1405β-con and M2297β-con, respectively, selected with Diagnostic Sensitivity Test agar (Oxoid, Basingstoke, Hampshire, United Kingdom) containing imipenem (8 μg/ml). M1405β-def D2$^-$ and M2297β-def D2$^-$ were β-lactamase basal mutants of M1405β-con D2$^-$ and M2297β-con D2$^-$. They were derived by mutagenesis with ethyl methanesulfonate, by the method described previously (1).

Outer membranes were prepared by the method of Anwar et al. (2) from logarithmic-phase cultures grown in Antibiotic No. 3 Broth (Difco, Detroit, Mich.). The protein concentration was measured by the method of Lowry et al. (11) and adjusted to 2 mg/ml. The extracts were electrophoresed on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system advocated by Hancock and Carey (7). The β-lactamase activity of cell sonicates was measured by spectrophotometric assay with 0.1 mM nitrocefin as the substrate (10). As required, the cultures were treated with cefoxitin (500 μg/ml) (Merck Sharp and Dohme, Hoddesdon, Hertfordshire, United Kingdom) for 2 h as a β-lactamase inducer.

MICs of antibiotics were determined with Diagnostic

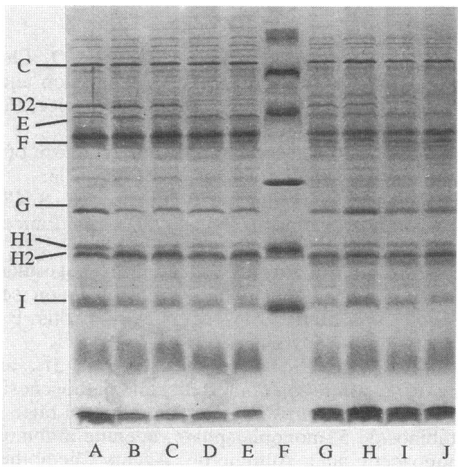

FIG. 1. Outer membrane protein profiles of *P. aeruginosa* strains and mutants. Lanes: A, M2297P; B, M2297β-con; C, M2297β-def; D, M2297β-con D2$^-$; E, M2297β-def D2$^-$; G, M1405β-con; H, M1405β-def; I, M1405β-con D2$^-$; J, M1405β-def D2$^-$. Lane F contains molecular weight markers (Bio-Rad, Richmond, Calif.) as follows (molecular weights are in parentheses): phosphorylase *b* (96,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (24,500), and lysozyme (14,400). The nomenclature of the outer membrane proteins, listed to the left, follows that of Hancock and Carey (7).

TABLE 1. β-Lactamase activities and antibiotic susceptibilities of *P. aeruginosa* mutants

Organism	β-Lactamase activity (nmol of nitrocefin hydrolyzed/min/mg of protein)		MIC (μg/ml)			
	Uninduced	Cefoxitin induced	Imipenem	Meropenem	Carbenicillin	Ceftazidime
M1405β-con	8,660	14,800	2	0.25	256	64
M1405β-def	3.1	1.1	0.25	0.12	128	2
M1405β-con D2$^-$	13,305	13,440	16	4	256	64
M1405β-def D2$^-$	10.9	6.3	0.5	2	128	2
M2297P	20.1	2,130	1	0.25	64	1
M2297β-con	9,220	4,950	1	0.25	128	32
M2297β-def	2.6	2.9	0.12	0.25	32	1
M2297β-con D2$^-$	15,400	13,700	16	2	64	32
M2297β-def D2$^-$	21.8	25.0	0.5	1	32	1

Sensitivity Test agar with inocula of 10^4 CFU per spot. The results were read after overnight incubation at 37°C. The compounds tested were carbenicillin (SmithKline Beecham, Brentford, Middlesex, United Kingdom), ceftazidime (Glaxo, Greenford, Middlesex, United Kingdom), chloramphenicol (Parke-Davis, Pontypool, Gwent, United Kingdom), imipenem (Merck Sharp and Dohme), meropenem (ICI, Macclesfield, Cheshire, United Kingdom), nalidixic acid, and tetracycline (Sigma Chemical Co., St. Louis, Mo.).

Confirmation of mutant phenotypes. SDS-PAGE confirmed the presence of the D2 protein, with a molecular weight of 54,000, in M1405β-con, M1405β-def, M2297P, M2297β-con, and M2297β-def and its absence from M1405β-con D2$^-$, M1405β-def D2$^-$, M2297β-con D2$^-$, and M2297β-def D2$^-$ (Fig. 1). Strain M2297 was confirmed to be inducible for β-lactamase, whereas the β-con organisms had high levels of uninducible enzyme and the β-def organisms had low levels of uninducible enzyme (Table 1).

Antibiotic susceptibilities of mutants. Among the organisms that expressed the D2 porin, imipenem MICs for β-lactamase-inducible strains and their derepressed mutants were equal, whereas β-lactamase basal mutants were eightfold more susceptible (Table 1). The β-con D2$^-$ mutants were eightfold more resistant to imipenem than their β-con D2$^+$ parents, but this resistance was lost in the β-def D2$^-$ mutants, which were only twofold less susceptible than the β-def D2$^+$ organisms.

Meropenem MICs for porin D2-producing organisms were 0.12 to 0.25 μg/ml, regardless of their mode of β-lactamase expression. Meropenem MICs for the β-con D2$^-$ organisms were raised 8- to 16-fold compared with those for their β-con D2$^+$ parents. Loss of the β-lactamase from the D2 porin-deficient organisms caused only a twofold increase in meropenem susceptibility. In keeping with previous results (13), the loss of the D2 protein was found not to affect susceptibility to carbenicillin and ceftazidime, which cannot pass through the channels formed by this porin (16). β-Lactamase derepression caused resistance to ceftazidime but not to carbenicillin, which is very stable to the β-lactamase of *P. aeruginosa* (10). MICs of chloramphenicol, nalidixic acid, and tetracycline were constant within each mutant series (not shown), contraindicating any change in and broad-spectrum permeability of the bacteria.

Overall, the present results show that imipenem resistance in *P. aeruginosa* required the chromosomal β-lactamase, as well as the loss of the D2 porin to which it is widely ascribed; mutants that lacked both the enzyme and the porin were almost as susceptible to imipenem as those that lacked the enzyme but retained the porin. Carbapenem resistance based on the interplay of the chromosomal β-lactamase and porin loss has also been described for *Enterobacter cloacae* and *Proteus rettgeri* mutants (14). Resistance in *E. cloacae*, however, required an exceptionally high level of β-lactamase, above that normally seen in derepressed mutants (14).

It is notable also that the ratio of imipenem MICs for the β-con and β-def organisms in the *P. aeruginosa* mutant series was 8 when the D2 porin was expressed, compared with 32 when the porin was absent. Thus, the β-lactamase gave greater protection in the less permeable background. This behavior conforms to the accepted theory on the interplay of impermeability and β-lactamase in gram-negative bacteria (12) but contrasts with the interaction of β-lactamases with intrinsic resistance in *P. aeruginosa* (9). Intrinsic resistance affects susceptibilities to penicillins, cephalosporins, quinolones, tetracycline, and chloramphenicol and is believed to entail broad-spectrum impermeability, though the precise mechanism remains uncertain. Surprisingly, β-lactamase production conferred the greatest increase in resistance to substrate β-lactams in the least intrinsically resistant (most permeable) isolates (9).

The present findings suggest that the loss of the D2 porin alone should not cause resistance to a carbapenem that was more stable than imipenem to the β-lactamase. Meropenem approached this condition, with its MICs being affected very little by the mode of β-lactamase expression in either the D2$^+$ or D2$^-$ background. Relating these observations directly to studies on the hydrolysis kinetics of carbapenems is difficult, as meropenem, unlike imipenem, can deactivate the pseudomonal β-lactamase, such that its hydrolysis rate declines more rapidly than is explicable by substrate depletion (17). Nevertheless, it appeared from the MICs for the mutants that the only functional defense against meropenem in β-lactamase-producing, D2$^-$ organisms was the porin loss, and this factor alone, unlike the combination of β-lactamase and impermeability acting against imipenem, could not raise the MICs beyond the clinical range.

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