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IMP-4, a Novel Metallo-β-Lactamase from Nosocomial *Acinetobacter* spp. Collected in Hong Kong between 1994 and 1998

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Between 1994 and 1998, 97 imipenem-resistant *Acinetobacter* isolates were identified at the Prince of Wales Hospital, Hong Kong, China. A bla_{IMP} PCR product was obtained from 23 of 35 viable cultures; 12 isolates belonged to genomic DNA group 3, 8 belonged to group 2 (*Acinetobacter baumannii*), 2 belonged to group 13TU, and 1 belonged to group 1. The bla_{IMP} homologues were sequenced from two isolates from genomic DNA group 2 and one isolate each from groups 3 and 13TU. The four sequences included an identical 738-bp open reading frame, predicted to encode a polypeptide of 246 amino acids, with 95.6% homology to IMP-1 and 89.3% homology to IMP-2. The new enzyme, designated IMP-4, was partially purified. It had a pI of 8.0 and was strongly active against imipenem and meropenem, with V_{max} values 53 and 8% of that for penicillin G, respectively. Strong activity was also seen against oxyimino-aminothiazolyl cephalosporins but not against aztreonam. Hydrolytic activity was inhibited by EDTA but not by clavulanate or tazobactam. Carbapenem MICs for most bla_{IMP}-positive isolates were 4 to 32 μg/ml, but one isolate with the intact gene was susceptible, with imipenem and meropenem MICs of 0.25 and 0.5 μg/ml, respectively. The latter isolate did not produce the band with a pI of 8.0, and gene expression was inferred to have been lost. None of the isolates studied in detail contained extrachromosomal DNA, and carbapenem resistance was not transmissible to *Escherichia coli*. Nevertheless, the presence of bla_{IMP-4} in different genomic DNA groups implies horizontal transfer, and sequences resembling a GTTRRRY integrase-dependent recombination motif were identified in the flanking regions of bla_{IMP-4}.

Acinetobacter spp. are important opportunistic pathogens, with *Acinetobacter baumannii* being the predominant species in clinical settings. Infection can involve virtually any body site in compromised patients, but acinetobacters are particularly associated with invasion of burn wounds and with nosocomial pneumonias in ventilated patients (5). The therapy of *Acinetobacter* infections is complicated by multidrug resistance: aminoglycosides, extended-spectrum cephalosporins, and fluoroquinolones were active against many *Acinetobacter* isolates during the early 1980s, but many clinical isolates are now resistant. Carbapenems have retained better activity than other antimicrobial agents, and resistance to carbapenems is still rare (15). Nevertheless, reports of carbapenem resistance among *Acinetobacter* spp. are accumulating steadily, with three types of mechanisms being encountered. Most carbapenem-resistant acinetobacters have OXA-type β-lactamases with weak activity against carbapenems; such enzymes have been found in *A. baumannii* isolates from Argentina, Belgium, France, Kuwait, Scotland, Spain, and Singapore (1, 2, 3, 6, 11, 13, 24). Several of these enzymes have been sequenced and are found to form a subgroup among class D β-lactamases, presently comprising the OXA-23, -24, -25, -26, and -27 types (3, 6, 11). A smaller (or less reported) group of acinetobacters owe their carbapenem resistance to β-lactamase-independent mechanisms (8, 12, 31). Finally, resistance mediated by metallo-β-lactamases has been reported in acinetobacters from Cuba, Italy, and Japan (9, 23, 30). The enzyme from the Italian isolates, designated IMP-2, has 84.9% amino acid homology with IMP-1 (26), a metallo-β-lactamase that is scattered in *Pseudomonas aeruginosa*, *Serratia marcescens*, and acinetobacters in Japan and that has been recorded from single isolates of *Klebsiella pneumoniae* in Japan and Singapore (17, 21, 28, 29). We report here a further IMP-type β-lactamase that confers carbapenem resistance in acinetobacters collected at the Prince of Wales Hospital, Hong Kong, China, between 1994 and 1998.

MATERIALS AND METHODS

Strain selection and identification. Acinetobacters were obtained from clinical specimens at the Prince of Wales Hospital, Shatin, Hong Kong, and all those (n = 97) reported by the clinical diagnostic laboratory to be resistant to imipenem between September 1994 and October 1998 were selected. This categorization was based on the British Society for Antimicrobial Chemotherapy disk method (33) from 1994 to 1997 and on the NCCLS disk method (20) from January 1998 onward. Viable cultures were revived from nutrient agar slants, which had been stored at room temperature for up to 5 years. Transformation (16) was used to confirm the genus identification, and genomic DNA groups were determined by amplified 16S rRNA gene restriction analysis (ARDRA) (10).

Antibiotics and susceptibility tests. MICs were determined by the British Society for Antimicrobial Chemotherapy's agar dilution method (33). The sources of the antibiotics were as follows: ampicillin, benzylpenicillin, cephalo-

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TABLE 1. MICs for bla_{IMP-4}-positive isolates of *Acinetobacter* spp.

Isolate type and lab no.	Sample	DNA group	Date isolated (mo/yr)	β-Lactamase pI(s)	MIC (μg/ml)[a]						
					IMP	MEM	ATM	PIP	TZP	CTX	SUL
Isolates from which bla_{IMP-4} was sequenced											
116665	Blood	2	10/94	5.4	0.5	0.25	16	>128	32	>128	16
127091	Blood	2	9/96	7.6, 8.0[b]	16	16	64	>128	64	>128	4
74510	Wound	13TU	5/95	5.7, 7.6, 8.0[b]	>32	>32	32	>128	32	>128	16
104680	Blood	3	8/98	5.7, 7.6, 8.0[b]	8	16	32	>128	64	>128	1
Collection of 23 bla_{IMP-4} positive isolates											
MIC range					0.25–16	0.25–16	2–64	64–256	4–64	32–256	1–64
MIC_{50}[c]					4	8	32	256	32	128	2
MIC_{90}[d]					16	16	64	256	64	256	16

[a] ATM, aztreonam; CTX, cefotaxime; IMP, imipenem; MEM, meropenem; PIP, piperacillin; SUL, sulbactam; TZP, piperacillin-tazobactam (tazobactam at 4 μg/ml).
[b] Enzymes with pIs of 8.0 ceased to be detectable if the gel was overlaid with 3 mM EDTA for 10 min before addition of nitrocefin.
[c] MIC_{50}, MIC at which 50% of isolates are inhibited.
[d] MIC_{90}, MIC at which 90% of isolates are inhibited.

ridine, cephalothin, nalidixic acid, oxacillin, and rifampin, Sigma Chemical Co. (St. Louis, Mo.); aztreonam, Bristol-Myers Squibb (Syracuse, N.Y.); cefotaxime, Aventis (Wembley, United Kingdom); ceftazidime and cefuroxime, GlaxoWellcome (Stevenage, United Kingdom); clavulanic acid, SmithKline Beecham (Brentford, United Kingdom); imipenem, Merck Sharp & Dohme (Hoddesdon, United Kingdom); meropenem, AstraZeneca (Macclesfield, United Kingdom); nitrocefin, BBL (Cockeysville, Md.); piperacillin and tazobactam, Wyeth (Taplow, United Kingdom); and sulbactam, Pfizer (Sandwich, United Kingdom).

Detection of bla_{IMP}-related sequences. PCR was used to detect bla_{IMP}-related sequences. Each reaction mixture (50 μl) contained 2 μl of genomic DNA, prepared by boiling a single colony in 200 μl of sterile distilled water; 25 pmol of each of the two primers (5′-ATG AGC AAG TTA TCT GTA TTC T [IMP11] and 5′-AGT GTG TCC CGG GCC ACC [IMP12]); 0.5 U of *Taq* DNA polymerase (Amersham-Pharmacia Biotech, Uppsala, Sweden); and the reaction buffer, supplied by the manufacturer. The mixtures were heated to 94°C for 2 min and were then subjected to 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final extension for 3 min at 72°C.

Cloning and sequencing of the bla_{IMP} open reading frame. Primers 5′-ATC CAA GCA GCA AGC GCG TTA (IMP13) and 5′-AGG CGT GCT GCT GCA ACG ACT TGT (IMP14) were used to amplify an 879-bp fragment containing the entire bla_{IMP} gene. The PCR conditions were those used for detection of bla_{IMP} (see above). The fragment was then subcloned into the expression vector pT-Adv with an AdvanTage PCR cloning kit (CLONTECH Laboratories, Palo Alto, Calif.). Recombinant plasmids were purified from selected clones with alkaline lysis minipreps (27). The DNA sequences and orientations of the inserts in these plasmids were determined on an ALFexpress DNA sequencer (Amersham Pharmacia Biotech) by using a cycle sequencing kit (SequiTherm Excel II; Epicentre Technologies, Madison, Wis.). The Cy-5-labeled M13 universal and reverse sequencing primers were used.

Plasmid detection, transfer, and curing. Plasmids were extracted for electrophoresis by alkaline lysis or by boiling of the minipreps (27). Conjugative plasmid transfer was attempted by plate mating with *Escherichia coli* K-12 J53-1 *pro* Nal^r or J53-2 *pro* Rif^r as the recipients, with counterselection on Diagnostic Sensitivity Test Agar (Oxoid, Basingstoke, United Kingdom) containing imipenem (1 μg/ml) plus nalidixic acid or rifampin (250 μg/ml), as appropriate. Curing was attempted by growing cultures in the presence of ethidium bromide at 0.25 or 0.5 time the MIC, recovering the cells on nutrient agar plates, and replica plating onto Iso-Sensitest agar (Oxoid) with and without imipenem (2 μg/ml).

Isoelectric focusing. Crude cell extracts were prepared by sonicating the overnight growth from nutrient agar in 0.1 M phosphate buffer (pH 7.0) and were clarified by centrifugation at 12,000 × *g*. Electrofocusing was performed at 15 W of constant current on gels with a pH range of 3.5 to 10, prepared by the method of Livermore and Williams (19), or on Phastgels at pH 3.5 to 9.0 run by using on automated electrophoresis system (Phastsystem; Amersham Pharmacia Biotech, Milton Keynes, United Kingdom). β-Lactamase bands were located with 0.5 mM nitrocefin. In some experiments duplicate gels were run, one of which was overlaid with 3 mM EDTA for 10 min before being developed with nitrocefin.

Purification and characterization of IMP-4 β-lactamase. Isolate 74510 was used as a source of β-lactamase for kinetic studies. Logarithmic-phase cells were harvested from 10 liters of nutrient broth culture, washed, and resuspended in 10 mM sodium phosphate buffer (pH 6.8) and were then disrupted by three passes through a French pressure cell (SLM Aminco, Urbana, Ill.) at 12,000 lb/in^2. After ultracentrifugation at 100,000 × *g* to remove the debris, the supernatant was loaded onto a column (40 by 2.6 cm) of carboxymethyl Sephadex C-50 equilibrated with 10 mM sodium phosphate buffer (pH 6.8). This was washed with the equilibration buffer and was then eluted with a linear gradient of 0 to 0.5 M NaCl, also prepared in 10 mM sodium phosphate buffer (pH 6.8). Eluent fractions were screened for activity against 0.1 mM imipenem by spectrophotometry at 297 nm and were subjected to isoelectric focusing. Imipenem-hydrolyzing fractions containing a single β-lactamase band were retained at −20°C. Enzyme activity was assayed by spectrophotometry at 37°C in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 mM $ZnCl_2$. The assay wavelengths were those listed by Livermore and Williams (19), and the kinetic parameters were calculated from Hanes plots (*s*/*v* versus *s*) of initial velocity data (*v*) obtained with 10 or more different substrate (*s*) concentrations. Inhibition assays with clavulanate, tazobactam, and disodium EDTA were performed under conditions in which either (i) the enzyme was added to a mixture of inhibitor and 1 mM benzylpenicillin or (ii) the inhibitor and enzyme were incubated for 10 min before addition of benzylpenicillin to a concentration of 1 mM.

Nucleotide sequence accession number. The nucleotide sequence containing the bla_{IMP-4} open reading frame has been assigned the EMBO/GenBank accession number AF244145.

RESULTS

Confirmation of resistance and detection of bla_{IMP}. Between September 1994 and October 1998, the clinical diagnostic laboratory reported 97 *Acinetobacter* isolates resistant to imipenem on the basis of the results of disk diffusion tests. Of these isolates, 35 remained viable and 23 gave 474-bp PCR products with the primers IMP11 and IMP12. By ARDRA, 12 of the 23 bla_{IMP}-positive isolates were found to belong to genomic DNA group 3, eight were found to belong to group 2 (*A. baumannii*), two were found to belong to group 13TU, and one was found to belong to group 1 (*Acinetobacter calcoaceticus*). The MICs for the bla_{IMP}-positive isolates are summarized in Table 1: one isolate (isolate 116665, Table 1) was fully susceptible to both imipenem and meropenem; the others were resistant or had reduced susceptibility compared with the susceptibilities of typical acinetobacters. Of the 12 PCR-negative isolates, 5 isolates were nevertheless confirmed to be imipenem resistant by NCCLS disk diffusion tests (20), whereas resistance was not confirmed for the remaining seven isolates.

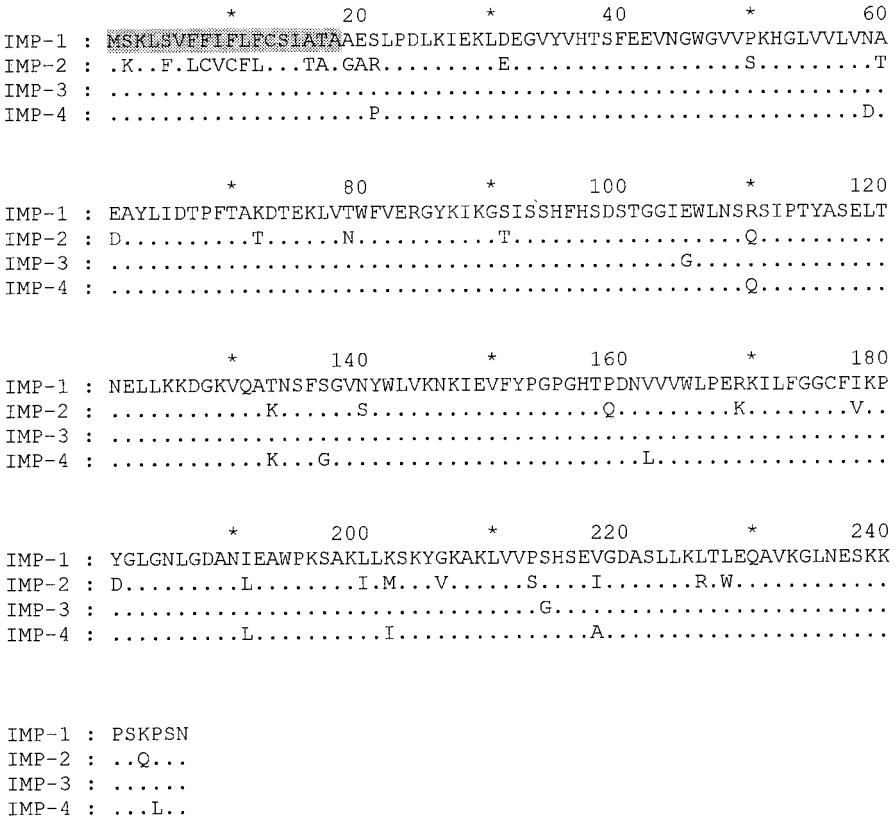

FIG. 1. Comparison of the amino acid sequences of IMP-1, IMP-2, IMP-3, and IMP-4 β-lactamases. The 18 residues that comprise the IMP-1 signal peptide (21) are shaded.

Sequencing of *bla*$_{IMP}$ homologues. An 879-bp fragment was cloned from four representative PCR-positive isolates (Table 1). These were selected (i) as two representatives from genomic DNA groups 2 and one each from DNA groups 3 and 13TU and (ii) as being from 4 of the 5 years in which PCR-positive isolates were collected. Three of the organisms were resistant to imipenem and meropenem, whereas one (isolate 116665) was the highly susceptible organism mentioned earlier. The cloned fragments from each of the four isolates were identical and contained the entire *bla*$_{IMP}$-related open reading frame and its flanking DNA. This open reading frame comprised 738 bases with 95.5% nucleotide identity to the *bla*$_{IMP-1}$ nucleotide sequence, as represented by the published sequences for *P. aeruginosa* (18) and *S. marcescens* (21). A total of 31 base differences from *bla*$_{IMP-1}$ were identified. These translated into 10 substitutions in the deduced amino acid sequence, designated IMP-4 (Fig. 1). The amino acid replacements reflected 11 of the base changes, whereas 20 further base changes were silent. A total of 96 nucleotide differences (13.0% divergence) and 37 amino acid differences (15.0% divergence) were observed compared with the nucleotide sequence of *bla*$_{IMP-2}$ and the amino acid sequence of its protein product, IMP-2 (Fig. 1). However, 10 of the differences from the IMP-2 protein lay in the putative signal peptide, which comprises the first 18 amino acids in the IMP-1 β-lactamase (21).

A GTTRRRY motif, also present upstream of *bla*$_{IMP-1}$ in *S. marcescens* isolates (4), was similarly seen upstream of the four *bla*$_{IMP-4}$ sequences and may be involved in integrase-dependent recombinations (see Discussion).

Plasmid transfer, detection, and curing. Both the alkaline lysis and the boiling miniprep methods failed to detect plasmids in any of the four strains used as sources of DNA for sequencing. Resistance was not conjugatively transferred to *E. coli* K-12 derivatives, and attempts to cure resistance with ethidium bromide were unsuccessful.

β-Lactamase characterization. Electrofocusing was performed with extracts of the four isolates from which *bla*$_{IMP-4}$ was sequenced. All except strain 116665 yielded bands with pIs of 8.0 that ceased to be detectable if the gels were overlaid with 3 mM EDTA for 10 min before nitrocefin was added as the reporter substrate and which therefore were deduced to be zinc dependent (Table 1). Isolates 104680 and 74510 additionally had β-lactamases with pIs of ca. 5.7 and 7.6; isolate 127091 had a β-lactamase with a pI of ca. 7.6, and isolate 116665 had a β-lactamase with a pI of 5.4. These enzymes with pI values of 5.4, 5.7, and 7.6 were not inhibited by EDTA.

On the basis of these inhibition experiments it was deduced that the band with a pI of 8.0 corresponded to IMP-4, and this enzyme was purified from isolate 74510. The final preparation was free of the β-lactamases with pIs of 5.7 and 7.6 produced by the isolate and contained only two major protein species when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One of these, which accounted for 80% of a total protein, had a molecular mass of ca. 30 kDa and was deduced to be IMP-4; the other was considerably smaller. This

TABLE 2. Kinetic properties of IMP-4 β-lactamase from isolate 74510

Substrate	Relative V_{max} (%)[a]	K_m (μM)
Benzylpenicillin	100	365
Ampicillin	52	575
Piperacillin	8	144
Carbenicillin	62	1,000
Oxacillin	307	420
Cephaloridine	81	32
Cephalothin	111	71
Cefuroxime	46	52
Cefotaxime	9	18
Ceftazidime	7	28
Imipenem	53	42
Meropenem	8	12
Aztreonam	0.001	Not measured

[a] Based on a predicted molecular mass of 27,087 and a purity of 80%; based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a k_{cat} of 27.5 s^{-1} was estimated for IMP-4 against benzylpenicillin.

preparation of IMP-4 enzyme had a very broad spectrum of activity (Table 2), encompassing penicillins, cephalosporins, and carbapenems. V_{max} values for imipenem and meropenem were 53 and 8% of those for benzylpenicillin, respectively, and hydrolysis of oxyimino-aminothiazolyl cephalosporins also was rapid, whereas aztreonam was stable. The 50% inhibitory concentrations of EDTA, clavulanate, and tazobactam were <0.1, 1, and 1.5 mM, respectively, when the inhibitor and enzyme were incubated together for 10 min before addition of 1 mM benzylpenicillin as the reporter substrate and <0.1, 0.85, and 2.7 mM, respectively, when no preincubation was allowed before addition of substrate.

Behavior of cloned *bla*$_{IMP-4}$ in *E. coli*. One *E. coli* TOP10F′ transformant, which resulted from the TA cloning of the 879-bp *bla*$_{IMP-4}$-coding fragment from isolate 104680, was tested for imipenem susceptibility. This organism had the insert correctly oriented with respect to the orientation of the *lac* promoter in the vector pT-Adv. The imipenem MIC rose from 0.38 μg/ml in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1.5 μg/ml in its presence, whereas IPTG had no effect on the imipenem MIC (also 0.38 μg/ml) for another transformant, which had the insert in the reverse orientation with respect to the orientation of the *lac* promoter. An analogous effect was seen for meropenem in disk diffusion tests, but this was not investigated in detail.

DISCUSSION

Carbapenem resistance in *Acinetobacter* spp. is an emerging concern and is disturbing because many nosocomial acinetobacters are already resistant to most other antibiotics (1, 5). The present isolates were from among carbapenem-resistant *Acinetobacter* spp. at the Prince of Wales Hospital, Hong Kong, that had been a long-standing (5-year) problem. Of 35 recoverable isolates reported to be imipenem resistant by the diagnostic laboratory, 23 had *bla*$_{IMP}$ homologues, 5 were confirmed to be imipenem resistant but lacked *bla*$_{IMP}$ and 7 lacked or had lost their resistance. Sequencing of the *bla*$_{IMP}$ homologues from four representative PCR-positive isolates revealed that they had *bla*$_{IMP-4}$, a new variant in the growing *bla*$_{IMP}$ family. The imipenem-resistant isolates that failed to give PCR products remain to be investigated further.

After discounting the 18 N-terminal residues, which are believed to constitute a signal peptide (21), the deduced sequence for the mature *bla*$_{IMP-4}$ product had 95.6% amino acid homology to the amino acid sequence of the IMP-1 enzyme and 89.3% homology to that of the IMP-2 enzyme. The six residues that are believed to hold zinc ions in the active center of metallo-β-lactamases (His 95, His 97, Asp 99, His 157, Cys 176, and His 215 [22]) were conserved in the IMP-4 enzyme as well as in the IMP-1, -2, and -3 enzymes. The signal peptides of IMP-1 and -4 were also deduced to be identical, although *bla*$_{IMP-4}$ had a silent C→T change at codon 13. IMP-2 has a substantially different signal peptide, with 10 amino acid substitutions compared with the sequences of both IMP-1 and -4 (26). Although all these data indicate that IMP-4 is closer to IMP-1 than to IMP-2, the new enzyme did share some of the amino acid changes that distinguish IMP-2 from IMP-1, specifically, Arg(110)→Gln, Thr(133)→Lys, and Ile(191)→Leu. IMP-3, which was recently described from *Shigella flexneri* in Japan, is a minor variant of IMP-1 with two amino acid substitutions (14).

The four isolates from which the sequence of *bla*$_{IMP-4}$ was confirmed were collected in 1994, 1995, 1996, and 1998, indicating long-term stability. They included representatives of genomic DNA groups 2, 3, and 13TU (Table 1), indicating horizontal spread; nevertheless, none of the isolates contained detectable plasmids, and none could transfer carbapenem resistance by conjugation. It is therefore inferred that *bla*$_{IMP-4}$ had become chromosomally integrated. Significantly, a GTTRRRY motif that is known to be involved in integrase-dependent recombination was found upstream of the *bla*$_{IMP-4}$ reading frame, and a related sequence was identified downstream (data not shown). The same sequence was previously found in *bla*$_{IMP-1}$-carrying elements from *S. marcescens* in Japan (4, 18). Transfer of *bla*$_{IMP}$-encoding integrons among *Acinetobacter* strains, with subsequent chromosomal integration, is therefore a plausible explanation for the spread of these genes. *bla*$_{IMP-1}$ likewise is often nontransmissible (28) and is inferred to become chromosomally inserted. Takahashi et al. (30) have recently reported the transfer of a *bla*$_{IMP}$ determinant, possibly on a plasmid, to an *Acinetobacter* isolate which had lost the gene on storage.

Multiple sets of kinetic data have been published for the IMP-1 enzyme, with relative V_{max} (k_{cat}) rates for ampicillin, cephaloridine, and imipenem variously reported as 100, 6, and 5, respectively (18); 100, 30, and 7, respectively (21); and 100, 46, and 11, respectively (32). This scatter may reflect assay conditions rather than fundamental differences among the IMP enzymes. Nevertheless, it is evident that IMP-4, like IMP-1, -2, and -3, hydrolyzes imipenem more rapidly than it hydrolyzes meropenem, has strong activity against oxyimino-aminothiazolyl cephalosporins and carboxy- and amino-penicillins, but spares monobactams (14, 18, 21, 32).

Expression of resistance correlated imperfectly with carriage of *bla*$_{IMP-4}$. Of 23 isolates positive for *bla*$_{IMP}$ by PCR, MICs were at least 4 μg of meropenem per ml for 22 isolates and at least 4 μg of imipenem per ml for 20 isolates. One isolate, however, was inhibited by these carbapenems at 0.25 or 0.5 μg/ml and thus was no less susceptible than *Acinetobacter* iso-

lates without carbapenem-hydrolyzing β-lactamases (25). This isolate (isolate 116665, Table 1) was among the four from which bla_{IMP-4} was sequenced, and since no activity of the β-lactamase with a pI of 8.0 was detectable on isoelectric focusing, it is deduced that the organism had little or no expression of its bla_{IMP-4} gene. Carbapenem susceptibility is also observed in some bla_{IMP-1}-positive *P. aeruginosa* isolates from Japan (28, 29), but it is not clear whether this behavior is because resistance demands secondary changes to permeability (via the loss of OprD in the case of *P. aeruginosa*) or because bla_{IMP-1} is not always expressed. Cloned bla_{IMP-4} gave only a very low level of imipenem resistance in *E. coli*, even when it was linked to a *lac* promoter and induced with IPTG. Similar results were obtained with cloned bla_{-IMP-1} (21), and it seems that IMP enzymes confer carbapenem resistance only in members of the family *Enterobacteriaceae* with concomitant permeability lesions.

Although the origins of the growing family of IMP β-lactamases remain uncertain, it is evident that multiple *Acinetobacter* lineages with bla_{IMP-4} have been prevalent at the Prince of Wales Hospital for a protracted period. This, taken together with the growing worldwide catalogue of reports of carbapenem-resistant acinetobacters, presents a disturbing situation. Clinicians treating infections caused by these organisms are forced to use ampicillin-sulbactam or cefoperazone-sulbactam so as to exploit the inherent activity that sulbactam has against many *Acinetobacter* strains (7, 31) or to use polymyxins, which are almost universally active against *Acinetobacter* spp. in vitro but which have questionable clinical efficacy.

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