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Pseudomonas pseudomallei Resistance to β-Lactam Antibiotics Due to Alterations in the Chromosomally Encoded β-Lactamase

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***Pseudomonas pseudomallei*, the causative agent of melioidosis, is generally susceptible to some of the newer extended-spectrum cephalosporins or to combinations of a β-lactam and clavulanic acid, a β-lactamase inhibitor. Resistance to these agents may, however, emerge during treatment. We report on alterations in the chromosomal β-lactamase associated with the development of resistance. Three resistance patterns resulted from three different mechanisms in the strains investigated. Derepression of the chromosomal enzyme resulted in a general increase in the MICs of all of the β-lactams tested. The second mechanism observed was an insensitivity to inhibition of the β-lactamase by clavulanic acid. In this case, the level of susceptibility to β-lactams as independent entities remained unchanged. The final "resistance" pattern occurred in a patient treated with ceftazidime and resulted in a β-lactamase that was capable of hydrolyzing this antibiotic at detectable levels, but with reduced efficacy against other β-lactams. The net result was a strain that was generally susceptible to all of the β-lactams tested except ceftazidime. In all cases, the level of susceptibility to antibiotics other than β-lactams remained unchanged. Such variability found within one genus over a relatively short time course suggests that treatment of infections caused by this organism should be carefully monitored to detect susceptibility alterations to the chosen therapy.**

Melioidosis is the name given to infections of humans or other animals caused by *Pseudomonas pseudomallei* (7, 18). The disease is endemic in southeastern Asia and northern Australia and is probably underdiagnosed in many other tropical regions (8). In parts of northeastern Thailand, melioidosis accounts for nearly 20% of cases of and 40% of deaths caused by community-acquired septicemia (5). *P. pseudomallei* may also cause localized infections in almost any body site (7, 18), while asymptomatic seroconversion is found in up to 50% of the population in endemic areas (17). The organism may remain latent for many years, causing recrudescent melioidosis at subsequent times of intercurrent stress (7, 18). The proportion of seropositive individuals who still harbor viable bacteria and are therefore at risk of recrudescent melioidosis is unknown.

P. pseudomallei is a gram-negative, saprophytic bacillus which has been isolated from both surface water and soil in endemic areas (33, 36, 37) and may persist in soil for prolonged periods. The organism is intrinsically resistant to many antimicrobial agents, including aminoglycosides and penicillins (10, 12). However, several of the newer β-lactams, including extended-spectrum cephalosporins such as ceftazidime and the combination of amoxicillin with the β-lactamase inhibitor clavulanic acid, are active at therapeutically achievable concentrations (6, 10, 12, 32). Ceftazidime has recently been shown to halve the mortality of acute, severe melioidosis compared with that after conventional, multiagent chemotherapy (41), and amoxicillin-clavulanic acid has given promising results in a preliminary clinical evaluation (39a; unpublished data). One problem which has emerged during clinical studies has been the development of resistance to the antibiotics in use (9, 10). Here we report alterations in the resident chromosomal β-lactamase in three resistant isolates of *P. pseudomallei* exhibiting three distinct patterns of resistance.

MATERIALS AND METHODS

The strains used in this study, their isolation sites, and their dates of isolation are given in Table 1. Clinical data for the patients have been reported elsewhere (9). In each case, one susceptible and one resistant isolate from each of the three patients were examined. β-Lactam resistance was defined as an MIC of >32 μg/ml for ceftazidime, amoxicillin-clavulanic acid, or both, determined as described previously (10). All culture handling was performed in an isolation room in a class 2 biological safety cabinet.

Growth conditions and cellular disruption. *P. pseudomallei* cultures were grown overnight in brain heart infusion broth (Difco) at 37°C. These cultures were used as starter sources for daily broth cultures or, in some cases, were used as the source of β-lactamase. Normally, enzymatic activity was determined from logarithmic-phase cultures obtained by inoculating a 1:10 dilution of the overnight culture into fresh brain heart infusion broth and continuing the incubation for 3 to 4 h prior to harvesting the cells.

Cells were harvested by centrifugation, in sealed bottles, at 5,000 rpm and 4°C in a Beckman centrifuge. Cells were washed in 0.89% saline and collected by centrifugation. Washed cells were resuspended in 0.05 M phosphate buffer (pH 7.4) and disrupted by sonication on ice (four 30-s pulses with 30-s cooling periods between bursts) or by five cycles of freeze-thawing (freezing to −70°C and rapidly thawing at 50°C constituted one cycle). The remaining intact cells and larger pieces of cellular debris were removed by centrifuga-

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TABLE 1. Strains used in this study

Patient no.[a]	Strain	Isolation date (mo-day-yr)	Isolation site	Treatment
1	316a	10-27-87	Blood	Ceftazidime
	316c	11-14-87	Blood	
2	365a	6-15-88	Urine	Amikacin-ceftazidime
	365c	6-15-88	Blood	
3	392a	7-21-88	Blood	Amoxicillin-clavulanate
	392f	11-15-88	Blood	

[a] Both isolates from patient 2 were obtained on the same day from different body sites. Patient 2 was treated with an unspecified β-lactam antibiotic for 2 days prior to admission. Patients 1 and 3 had no known history of β-lactam therapy prior to admission.

tion (3,000 rpm for 10 min), and the supernatant was collected as the source of β-lactamase. The supernatant was split into aliquots, assayed for protein content (20), and stored at −20°C until it was required for use.

β-Lactamase assays. Hydrolysis of selected β-lactams was monitored by a spectrophotometric assay (19) in 50 mM phosphate buffer (pH 7.4) at 37°C. K_m and V_{max} values were derived from analysis of Hanes (s/v versus s) plots of hydrolysis rates (v) derived at different substrate concentrations (s). The wavelengths used were the same as those quoted by Livermore et al. (19), as follows: carbenicillin and penicillin G, 235 nm; ceftazidime, 257 nm; cephaloridine, 295 nm; cefoxitin, 263 nm; cephalothin, 288 nm. PADAC (Glaxo) and nitrocefin were assayed at 570 and 482 nm, respectively, by the recommendations of their manufacturers. Amoxicillin was monitored at 227 nm. All substrates were resuspended and diluted in 50 mM phosphate buffer (pH 7.4).

The inhibitory effect of prebinding of penicillin G or clavulanic acid to the β-lactamase preparations prior to the addition of nitrocefin was calculated for all strains. Inhibitor at various concentrations was added to the preparations 5 min prior to the addition of 9.69 μM nitrocefin. Hydrolysis of nitrocefin was monitored over a 30-min period immediately following its addition.

When induced cultures were used as the enzyme source, cultures were exposed to 0.5 to 0.125× the MIC of the inducer for 3 h prior to harvesting. Imipenem, at 0.125× the MIC, was the inducer that was used most often, although cephaloridine, cephalothin, or penicillin G (all at 0.5× the MIC) was used for different experiments.

Penicillin-binding proteins. Total membrane preparations were labeled with ^{35}S-penicillin G (1.4 Ci/mmol; New England Nuclear Corp.) essentially as described previously (13). A total of 15 μg (50 μl) of membrane preparation was labeled with 30 μg (10 μl) of ^{35}S-penicillin G and incubated at room temperature for 10 min; the binding interaction was stopped by the addition of unlabeled penicillin (5 μl of penicillin at 120 mg/ml) in gel loading buffer (13) and suspension in a boiling water bath for 10 min. The heated samples were loaded directly onto a sodium dodecyl sulfate-polyacrylamide gel and separated by electrophoresis at a 25-mA constant current until the marker dye neared the bottom of the gel. Gels were impregnated with a fluor (Amplify; Amersham) for 30 min, washed in water (20 min), dried, and exposed to prefogged Kodak XAR5 X-ray film at −70°C for up to 2 weeks before viewing.

Isoelectric focusing of β-lactamase. A total of 10 to 15 μg of protein of the sonically extracted cell-free supernatant centrifuged at 3,000 rpm was loaded onto filter paper discs and placed onto the surface of a horizontal polyacrylamide gel containing the following ingredients: acrylamide (7.5% [wt/vol]), *N,N'*-methylene bisacrylamide (0.2% [wt/vol]), glycerol (10%), Pharmalyte (pH 3.5 to 10 or pH 6 to 8; 6.7% [vol/vol]; Pharmacia Fine Chemicals, Uppsala, Sweden), ammonium persulfate (0.007% [wt/vol]), and *N,N,N',N'*-tetramethylethylenediamine (0.003% [wt/vol]). Thin gels (1 mm) were attached to glass plates during polymerization and were prefocused at 3 W of constant power for 30 min prior to use. Focusing was performed at 3 W of constant power for 5 h on an isoelectric focusing apparatus (Bio-Rad Laboratories, Richmond, Calif.). β-Lactamase was detected by overlaying the gel with either PADAC (100 μg/ml) or nitrocefin (100 μg/ml); both substrates were resuspended and diluted in 0.05 M phosphate buffer (pH 7.4).

Plasmid screening. The selected strains were screened for the presence of plasmids by electrophoresis of lysates, which were prepared by the alkaline lysis technique described by Maniatis et al. (21) or by the procedure of Hansen and Olsen (15), in 1% agarose gels and staining with ethidium bromide (21).

RESULTS

The MICs for the strains tested are given in Table 2. It is evident that several different resistance mechanisms may be acting in the three sets of strains. Strain 365a showed a high level of resistance to most of the β-lactams tested. This strain could not be induced to higher levels of β-lactamase activity when it was grown in the presence of sub-MICs of imipenem or cephaloridine (Table 3). These observations suggest that strain 365a may constitutively express β-lacta-

TABLE 2. MICs for β-lactam-resistant and -susceptible pairs of isolates of *P. pseudomallei*

Strain	MIC (μg/ml)[a]												
	Amox	Amox-Clav	Azlo	Azt	Ceft	Cefx	Ctx	Caz	Cro	Imp	Pip	Cip	Tet
316a	64	2	4	16	2	2	4	4	4	1	1	4	4
316c	32	1	2	4	4	4	8	64	4	0.25	1	4	4
365a	>256	>256	32	256	16	32	64	32	64	8	16	2	4
365c	64	2	2	16	1	2	4	1	4	2	1	2	4
392a	64	2	2	16	1	2	4	1	4	2	1	1	8
392f	64	32	2	32	2	4	4	1	4	2	1	1	8

[a] Amox, amoxicillin; Clav, clavulanic acid; Azlo, azlocillin; Azt, aztreonam; Ceft, cefetamet; Cefx, cefixime; Ctx, cefotaxime; Caz, ceftazidime; Cro, ceftriaxone; Imp, imipenem; Pip, piperacillin; Cip, ciprofloxacin; Tet, tetracycline.

TABLE 3. Induction of β-lactamase by subinhibitory concentrations of imipenem

Strain	Sp act[a]		Approximate fold increase
	Uninduced	Induced	
316a	70.7	354.3	5
316c	1.3	1.4	None
365a	463.5	471.3	None
365c	74.5	482.6	6
392a	51.0	411.1	5
392f	162.5	401.1	2.5

[a] Specific activity is expressed as micromolar cephaloridine hydrolyzed per minute per milligram of protein. Cells were induced for β-lactamase by growth of early-logarithmic-phase cultures in 0.5× the MIC of imipenem for 2 h prior to harvest. Numbers are means from four independent determinations. The largest standard deviation observed was 31.6 for induced strain 392a.

mase and that its resistance may be explained, at least in part, by this mechanism. Strains 316c and 392f exhibited narrower spectra of β-lactam resistance. Strain 316c was convincingly resistant only to ceftazidime, with a 16-fold decrease in susceptibility to this antibiotic. The other resistant strain, 392f, showed elevated resistance to β-lactamase inhibition by clavulanic acid, resulting in its ability to grow in the presence of amoxicillin or ticarcillin and the inhibitor.

The strains were examined for the presence of plasmid DNA by two isolation procedures, with negative results in all cases (data not shown).

All of the strains tested produced a β-lactamase which was detected in whole-cell sonic extracts without further purification or in freeze-thaw lysates. In lysates cleared of cells and larger bacterial debris by centrifugation at 16,000 × *g*, the β-lactamase activity was considerably less than that observed in the cruder preparations (data not shown), suggesting that the enzyme may be membrane associated. Kinetic data for the β-lactams tested are given in Table 4. Strain 316c was the only strain to give a detectable K_m for ceftazidime, while no activity was detected in the other strains with the enzyme-substrate combinations tested. Hydrolysis rates showed that the relative activity of strain 316c was reduced for the substrates tested, with the exception of ceftazidime and nitrocefin. The hydrolysis rates were determined for both uninduced (shown) and imipenem-induced cultures (Table 4). Upon induction, hydrolysis levels increased for strains 316a, 365c, 392a, and 392f but not for strain 316c or 365a, implying that the latter two strains were constitutive β-lactamase producers.

TABLE 4. β-Lactamase characteristics for the strains tested

Strain	V_{max} for Ceph[a]	Hydrolysis and affinity characteristics for the tested β-lactams[b]							
		Ceph		Caz		Amox		Nitr	
		K_m	V	K_m	V	K_m	V	K_m	V
316a	97.7	95	100	ND[c]	<0.1	208	0.2	63	1.2
316c	1.7	12	100	12	76	ND	<0.1	168	106
365a	676.8	91	100	ND	<0.1	159	23.4	96	20.7
365c	110.5	96	100	ND	<0.1	147	0.6	84	1.7
392a	117.7	204	100	ND	<0.1	121	0.5	109	0.5
392f	328.4	207	100	ND	<0.1	137	0.4	87	2.3

[a] The V_{max} for cephaloridine (Ceph) is expressed as micromolar per minute per microgram of protein.

[b] Ceph, cephaloridine; Caz, ceftazidime; Amox, amoxicillin; Nitr, nitrocefin. *V*, relative hydrolysis rate of the different β-lactams compared with that of cephaloridine, which was set at 100.

[c] ND, not detected. No hydrolysis could be detected for these compound-strain combinations at the enzyme concentrations used in the time allowed.

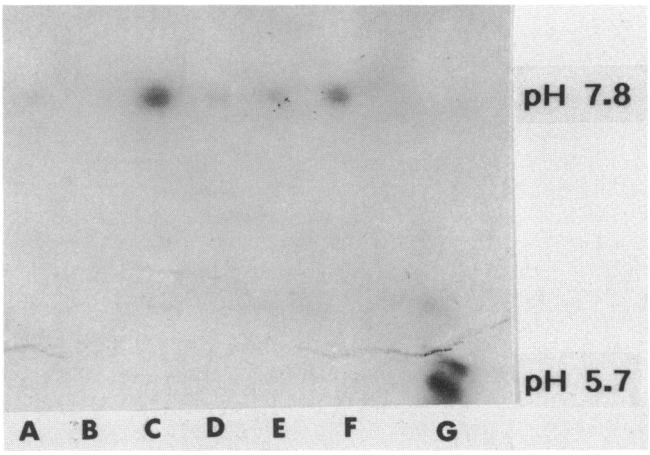

FIG. 1. Isoelectric focusing point for the three pairs of strains: 316a (lane A), 316c (lane B), 365a (lane C), 365c (lane D), 392a (lane E), and 392f (lane F). Whole-cell lysates were run on an isoelectric focusing gel with ampholytes (conferring a pH 5.5 to 8.5 gradient) incorporated; β-lactamase was detected by flooding the gel with nitrocefin. The pH was determined by screening one edge of the gel at set intervals with a surface electrode prior to flooding with nitrocefin. Lane G contains TEM-2 β-lactamase loaded as a control.

Examination of preparations on isoelectric focusing gels showed that only one band was capable of hydrolyzing nitrocefin (Fig. 1). This band was apparent in all of the preparations tested, although it was enhanced in strain 365a and diminished in strain 316c preparations, requiring overdevelopment to be clearly visible for the latter strain, and thus is not seen in Fig. 1. The β-lactamase focused at pH 7.4 to 7.8 on the gels, which is in agreement with the value previously established by Livermore et al. (19). The β-lactamase was detected following sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the procedure described by Tai et al. (35) (data not shown) and had an apparent molecular mass of between 25 and 29 kDa (Fig. 2), depending upon the gel system used. The amount of hydrolysis observed for strain 316c did not correlate with the amount of penicillin binding (Fig. 2) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis or with the amount of protein focused at this point on isoelectric focusing gels (data not shown). It appears that the enzyme in strain 316c is constitutive (Table 3), in that no more enzymatic activity was detected in cultures induced with 0.25× the MIC of imipenem or 0.5× the MIC of penicillin G or cephalothin.

The resistant strain 392f appeared to produce a β-lactamase which had moderate activity but which was reduced in its susceptibility to the inhibitory effects of clavulanic acid. Comparison of the inhibitory effects of clavulanic acid upon the hydrolysis of nitrocefin is shown in Fig. 3. When compared with the concentration required to inhibit the β-lactamase from the susceptible isolate (strain 392a), for the enzyme from strain 392f, more than a 10-fold increase in the concentration of clavulanic acid was required to produce a similar degree of inhibition. The resistance obtained appeared to be restricted to decreased susceptibility to β-lactams in the presence of clavulanic acid, with β-lactam antibiotic inhibitory concentrations in the absence of the

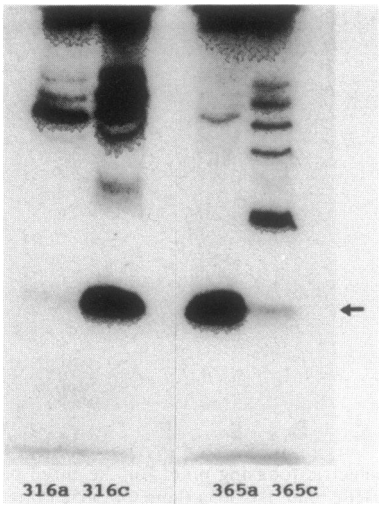

FIG. 2. Whole-cell lysates from strains 316a, 316c, 365a, and 365c labeled with ^{35}S-penicillin G in the presence of 10 μg of clavulanic acid per ml and detected by fluorography. The position of the β-lactamase is indicated by the arrow.

inhibitor remaining at the susceptible level (Table 2). The β-lactamase in this case was inducible to higher levels with induction by either imipenem (Table 3) or cephalothin (data not shown).

DISCUSSION

The results obtained with the enzyme preparations used in this study suggest that the β-lactamase activity is associated with the membrane fractions in all of the strains tested. This observation is consistent with that of Livermore et al. (19), who made a similar observation on the strains they tested. Treatment failure during therapy with newer β-lactams may result from several different alterations in the bacterial cell:

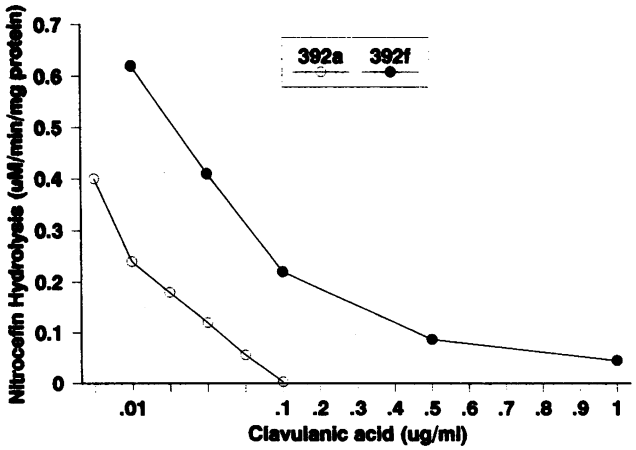

FIG. 3. Competition between clavulanic acid and nitrocefin for the β-lactamase from strains 392a and 392f. β-Lactamase-containing freeze-thaw lysate supernatants were preexposed to different concentrations of clavulanic acid and then to nitrocefin. The resultant hydrolysis of nitrocefin was monitored over time. The control hydrolysis rates in the absence of competitor were 1.72 ± 0.23 μM/min/mg of protein for strain 392a and 1.88 ± 0.17 μM/min/mg of protein for strain 392f.

the cell may be altered in the susceptibility of its targets, the cell may change its permeability characteristics to limit access to the cellular target, or the cell may produce an enzyme which inactivates the antibiotic before it reaches the target. Largely on the basis of the work of Livermore et al. (19), the β-lactamase of *P. pseudomallei* has been classified as a chromosomally produced oxyimino-cephalosporinase (28), which places it in the Richmond and Sykes class 1, group 1c, enzymes (27, 34) or in class 2e by the classification proposed by Bush (4). This class of enzyme tends to be less susceptible to inhibition by cloxacillin than it is to inhibition by clavulanic acid or sulbactam (14, 16, 19, 22, 23), which has been suggested as a means of counteracting the resistance caused by this enzyme class. Three different modifications of the chromosomal enzyme were shown in the present study to be present in β-lactam-resistant *P. pseudomallei*, resulting in three distinct patterns of β-lactam resistance: an apparent derepression of the enzyme, an altered substrate profile, and lack of susceptibility to β-lactamase inhibitors.

Derepression of the normally inducible enzyme, like that which occurred in strain 365a, is a mechanism commonly found in other pseudomonads and in some of the members of the family *Enterobacteriaceae* (2, 29–31, 39).

The observation of reduced efficiency for enzyme inhibitors is not unique. As reported by Thomson et al. (38), the level and type of β-lactamase produced by members of the family *Enterobacteriaceae* can result in resistance to the inhibitory effects of different inhibitors. In the case of strain 392f, it appears that the resident chromosomal enzyme was altered to give the insensitivity to inhibition by clavulanic acid, resulting in the reduced efficacy of the amoxicillin-clavulanic acid combination, with little effect on the activities of other β-lactams. This insensitivity was associated with changes in the enzyme level (Table 3), but without significant changes in the affinity of the β-lactamase for amoxicillin or other β-lactams (Table 4).

Resistance in the case of strain 316c appeared to be the result of an alteration in the specificity of the enzyme which allowed it to increase the rate at which it could hydrolyze ceftazidime (the antibiotic used for treatment in this case) but which resulted in reduced hydrolytic efficiency against the β-lactams which are normally good substrates for the enzyme.

Alteration of a Richmond and Sykes class 1 enzyme has been suggested as a mechanism by which bacteria may become resistant to newer antibiotics (28). Stable derepression (constitutive production) resulting in an increase in the number of β-lactamase molecules available to bind and either hydrolyze or "trap" the encroaching antibiotic has been suggested as the basis of reduced efficacy by the drug (28). In the case of the enzyme in strain 316c, constitutive production appeared to occur (Fig. 2), although significant changes in the level of resistance to drugs other than ceftazidime did not occur. This suggests that constitutive production per se is not the resistance mechanism in this strain, but that a change in the affinity of the enzyme appears to result in resistance. Weber et al. (40) described a derivative of TEM-1 (TEM-12) which is chromosomally encoded and mediates selective resistance to ceftazidime. The homology of TEM-12 with TEM-1, which is normally a plasmid-encoded enzyme, suggests that the chromosomal replication site for TEM-12 may have arisen via insertion of either a mutated TEM-1 or TEM-12 itself into the *Escherichia coli* chromosome. In the case of strain 316c, the chromosomal origin of replication appears to be the natural site, since all

strains of *P. pseudomallei* examined to date either express or can be induced to produce what appears to be the parental enzyme (19).

Plasmid-specified enzymes with the ability to hydrolyze ceftazidime have been described in *Klebsiella pneumoniae* (3, 24, 26) and other members of the family *Enterobacteriaceae* (1, 11, 25). However, most of the extended-spectrum β-lactamases that are capable of hydrolyzing ceftazidime maintain their original range of substrates and add others (25). The enzyme produced by strain 316c has a reduced substrate profile. The affinity for cephaloridine was increased (Table 4), although its hydrolysis was markedly reduced. This suggests that the enzyme in 316c is changed in both its hydrolytic and binding properties. It appears, then, that the β-lactamase of strain 316c is constitutive, is capable of hydrolyzing ceftazidime, and is able to bind penicillin G (Fig. 2). It is unlikely that constitutive production of the enzyme can confer this phenotype, since strain 365a (which is also constitutive) has the phenotype expected for this condition. A change in the substrate binding site of the enzyme would be a more likely mechanism by which the altered substrate hydrolytic profile and changed affinities could have arisen. The exact nature of the changes that occur in the strains described here will require a molecular characterization of the control mechanisms and the enzymes involved.

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