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Role of β-Lactamase in In Vivo Development of Ceftazidime Resistance in Experimental *Pseudomonas aeruginosa* Endocarditis

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Two ceftazidime-resistant variants of *Pseudomonas aeruginosa* (PA-48, PA-60), obtained from cardiac vegetations of rabbits with endocarditis receiving ceftazidime therapy, were studied for mechanisms of resistance. Both resistant variants were stably derepressed for the type Id β-lactamase, which was ceftazidime inducible in the parental strain (PA-96) used to initially infect the rabbits. There was no evidence of ceftazidime bioinactivation by the resistant strains, and their outer membrane permeabilities were comparable to those of the parental strain. No alterations were observed in patterns of outer membrane proteins or membrane lipopolysaccharides in the resistant variants as compared with the parental strain. Penicillin-binding protein patterns of the resistant variants revealed the absence of penicillin-binding protein 4 in both, with acquisition of a new protein of higher apparent molecular weight in PA-60. Calculation of the rate of appearance of ceftazidime in the periplasm at sub-MICs suggested that slow enzymatic hydrolysis of the β-lactam, rather than nonhydrolytic trapping, was the major explanation for the induced resistance in vivo in strains PA-48 and PA-60.

β-Lactam antibiotics are of primary use in the therapy of gram-negative bacterial infections. However, the appearance of bacterial resistance to successive generations of β-lactams (30, 36) has necessitated the continuous development and assessment of new compounds. The major impediments to β-lactam therapy have been the high intrinsic resistance of some organisms, including *Pseudomonas aeruginosa* due to the intrinsic barrier properties of their outer membranes (21, 39) and the acquisition of high levels of β-lactamase due either to the presence of a plasmid or to the derepression of a formerly inducible, chromosomally encoded β-lactamase (30, 34, 36). Mutational alterations in the β-lactam targets, penicillin-binding proteins (PBPs), have also been observed (26) but seem to occur with somewhat less frequency. One novel approach taken by pharmaceutical companies in the development of newer β-lactam antibiotics has been to search for compounds that are refractory to β-lactamase-mediated hydrolysis. Recently, however, it has been observed that, despite the extremely low rates at which these compounds are hydrolyzed, clinical isolates of *P. aeruginosa* and *Enterobacter cloacae* that are resistant to these so-called β-lactamase-stable β-lactams often have much-increased levels of the chromosomal β-lactamase (4, 30, 31, 34–36). The mechanism of resistance has been suggested to involve nonhydrolytic binding of the β-lactam by molecules of β-lactamase, thus reducing the periplasmic concentration (i.e., the concentration in the vicinity of the target PBPs) to subinhibitory levels (34). Alternatively, Vu and Nikaido (35) have argued convincingly that a combination of low-level hydrolysis by β-lactamase (low K_m, low V_{max}) and reduced outer membrane permeability, thus diminishing the rate of buildup of periplasmic β-lactam concentrations, is the actual mechanism of resistance.

Analysis of resistant clinical isolates presents a major problem, since it is difficult to obtain with certainty the control (parental) susceptible strain from which the resistant isolate arose. This makes characterization of antibiotic resistance, which may be multifactorial, problematic. In the current investigation, we have utilized an animal model infection to overcome these problems. The resistant isolates we studied arose during ceftazidime therapy of experimental *P. aeruginosa* endocarditis in rabbits (2). Our results confirm that strains with derepressed chromosomal β-lactamase production can arise during therapy and suggest that the overproduction of β-lactamase is the most significant cause of resistance.

MATERIALS AND METHODS

Bacterial strains. PA-96 was a clinical isolate of *P. aeruginosa* that was used to initially infect catheterized rabbits to induce experimental aortic valve endocarditis (2). Its identification, serotyping, and rabbit serum resistance have been previously described (2). Most β-lactams tested have moderate MICs for this strain (2), including ceftazidime (8 μg/ml), cefoperazone (16 μg/ml), and moxalactam (32 μg/ml). A detailed description of the induction, antimicrobial therapy, and timing of development of β-lactam resistances has been recently reported (2). Two ceftazidime-resistant variants (PA-48 and PA-60), utilized in the current investigations, were isolated directly from homogenates of cardiac vegetations subcultured onto agar containing ceftazidime (50 μg/ml). Both ceftazidime-resistant variants (MIC, >128 μg/ml) were cross resistant to cefoperazone (>64 μg/ml) and moxalactam (>64 μg/ml). In addition, these variants were stable in vitro, since after 15 serial passages in antibiotic-free

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TABLE 1. β-Lactamase activities of periplasmic extracts from strain PA-96 and its ceftazidime-resistant variants PA-48 and PA-60 from cardiac vegetations

Isolate	β-Lactamase activity[a]	
	Uninduced	Induced
PA-96	30	260
PA-83-48	8,850	7,400
PA-83-60	2,740	4,960

[a] Expressed as micromoles of cephalothin hydrolyzed per milligram of protein per minute.

media they grew well when plated onto MH agar containing ceftazidime (50 μg/ml).

β-Lactamase assays. Overnight cultures of strains PA-96, PA-48, and PA-60 grown in Mueller-Hinton or proteose peptone no. 2 broth were inoculated into fresh media at a 1:100 dilution and grown to an optical density of 0.5 to 1.0 at 600 nm. Cells were harvested by centrifugation and suspended in 10 mM sodium phosphate buffer (pH 7.5), and their periplasmic β-lactamases were released by freeze-thaw techniques (13) or by treatment of cells with polymyxin B (1 mg/ml) (10). In parallel experiments, growing cultures of the three pseudomonal strains were induced with a sub-MIC of ceftazidime (4 μg/ml), and the cells were grown for 3.5 h before harvesting and extraction of periplasmic β-lactamases, as described above. Protein contents of the periplasmic extracts were determined by the Bio-Rad technique. For cephaloridine and cephalothin hydrolysis, β-lactamase activities were measured by a spectrophotometric method at substrate concentrations of 100 μM (32). The nitrocefin chromogenic β-lactamase assay (27) was utilized in kinetic assays of possible ceftazidime hydrolysis at low substrate concentrations and for possible ceftazidime inhibition of β-lactamase activity through nonhydrolytic mechanisms. Varian DMS 200 and Perkin-Elmer Lambda 3 spectrophotometers were utilized in the β-lactamase kinetic assays; the slit widths utilized were 0.5 and 1.0 nm, respectively.

Detection of enzymatic modification of ceftazidime. Although ceftazidime is relatively resistant to β-lactamase hydrolysis (19), other periplasmic enzymes (e.g., acylases) elaborated by the ceftazidime-resistant variants might alter the drug's bioactivity. A modification of the technique of Burns et al. (5) was utilized to investigate this possibility. A lawn of a ceftazidime-susceptible *Escherichia coli* (ATCC 10536) was streaked on an MH agar plate. Disks of Whatman no. 1 filter paper, 8.5 mm in diameter, were placed on the *E. coli* lawn. A heavy inoculum of the pseudomonal strains (PA-96 and the two resistant variants) was applied evenly over the surface of separate filter papers, and a 67.5-μg ceftazidime disk was placed centrally on the inoculated filter papers. One filter paper was inoculated with sterile saline as a control. The plates were incubated overnight at 37°C, and the zones of inhibition were measured. The ceftazidime must pass unmodified through the filter paper inoculated with the pseudomonal strain to inhibit growth of the *E. coli*. The zone size should have been reduced (versus the parental strain) or absent if the ceftazidime was inactivated by the organism on the filter paper (5).

Characterization of β-lactamases by gel electrophoresis and isoelectric focusing. The periplasmic β-lactamase extracts from the parental and ceftazidime-resistant variants, prepared as described above, were electrophoresed under dissociating conditions by the method Laemmli (17) with 10% polyacrylamide slab gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]). Samples (75 μg) of protein were loaded per lane from each extract, and the resolved polypeptides were stained with Coomassie blue.

Isoelectric focusing was performed in duplicate under both native and denaturing conditions in a model 175 tube cell (Bio-Rad Laboratories) as described Benya (3), except that under native conditions urea was omitted from the loading buffer and gel. After focusing, the pH gradient was confirmed by sectioning the gels into 10 1-cm slices and eluting each slice separately into 2.0 ml of degassed KCl (0.10 M) in sealed vials. Native gels were activity stained for β-lactamase by overlaying tube gels with MH agar containing 50 μg of nitrocefin per ml. The appearance of pink bands within the gels localized β-lactamase activity (8).

Two-dimensional separations were performed with isoelectric focusing as the first dimension and SDS-PAGE as the second dimension as described by O'Farrell (25). Slab gels were stained for protein with silver stain.

Outer membrane permeability assays. Outer membrane permeability was assessed by a modification of the technique of Zimmerman and Rosselet (40) with nitrocefin as previously described (27). To allow direct comparisons of uninduced strains, the TEM-2 β-lactamase from plasmid R68.45 was introduced into strains PA-96 and PA-60 by conjugating these strains with *P. aeruginosa* H762 (an arginine auxotroph of *P. aeruginosa* PAO carrying plasmid R68.45), as previously described (21). Transconjugants of PA-96 and PA-60 carrying R68.45 were isolated on BM2 minimal succinate medium (9) by using the lack of arginine in the plates as a counterselection against the donor and 300 μg of kanamycin per ml or 500 μg of carbenicillin per ml as the selective agent. For outer membrane permeability studies, cultures were grown to an optical density at 600 nm of 0.5 to 0.8.

Cell envelope and PBP analyses. For lipopolysaccharide analyses, the method of Hitchcock and Brown (12) was used. Outer and inner membranes were isolated by utilizing differential sucrose gradients by the method of Hancock and Nikaido (9). PBP analyses of inner membrane proteins were performed after two cycles of suspension of inner membranes in 10 mM Tris hydrochloride (pH 8.0) and sonication followed by centrifugation at 150,000 × *g* for 1 h. This treatment was required to extract β-lactamase from inner membrane preparations (T. R. Parr, L. Chan, A. S. Bayer, and R. E. W. Hancock, submitted for publication). PBPs were analyzed as described by Spratt (33) with ^{35}S-labeled benzyl penicillin (New England Nuclear Corp., Boston, Mass.; 3.7 Ci/mmol; 12.5 μg of benzylpenicillin per ml, final concentration). SDS-PAGE was carried out as described previously (1).

RESULTS

β-Lactamase activity. Preliminary studies on French-pressed broken cells of PA-48 and PA-60 indicated that these variants had far greater basal levels of β-lactamase than the parental strain (PA-96). This was confirmed by analysis of the β-lactamase activities of the periplasmic extracts of these strains (Table 1). Both PA-48 and PA-60 had significantly higher levels of β-lactamase activities for both cephalothin (Table 1) and cephaloridine (data not shown) in the basal (uninduced) states than the parent strain (>90 to 300 times, respectively, for cephalothin). Induction of PA-96 by a sub-MIC of ceftazidime resulted in an approximately eightfold increase in β-lactamase activity over the uninduced

TABLE 2. Identification of the β-lactamase in strain PA-48 as the type Id chromosomal β-lactamase of *P. aeruginosa*

β-Lactamase	Inducibility	Mol wt	pI	K_m (μM) for:		K_i (μM) for ceftazidime
				Cepholoridine	Nitrocefin	
From PA-48	Constitutive	41,000[a]	6.95–8.1[b]	59.0 ± 9.8	18.6 ± 2.8	11.4 ± 1.3
Type Id	Inducible	41,000 to 42,000[a] (6)[c]	7.2–8.15 (6, 20, 38)	50 (6)	24.5 ± 4.7[d]	11 (16)

[a] Obtained by SDS-PAGE; gel chromatography usually resulted in lower values. Our own data suggested a molecular weight of 41,000 for the induced enzyme of strain PA-96.
[b] Three bands, activity stained with nitrocefin, with pIs of 6.95, 7.4, and 8.1 were obtained.
[c] Numbers within parentheses indicate references.
[d] T. R. Parr and R. E. W. Hancock, unpublished data; the PA-96 enzyme was induced with 200 μg of penicillin G per ml.

strain ($P > 0.01$). In contrast, the increased β-lactamase activities of strains PA-48 and PA-60 in the uninduced state could not be significantly induced further.

Following the guidelines of Vu and Nikaido (35), we attempted to demonstrate hydrolysis of ceftazidime at low substrate concentrations by periplasmic β-lactamase extracts of PA-48 or PA-96 (postinduction). Each of these extracts hydrolyzed nitrocefin, (as assayed by an increase in A_{520}) and cephaloridine (as assayed by a decrease in A_{260}). The K_m values obtained from these latter assays for nitrocefin and cephaloridine hydrolysis by strain PA-48 were consistent with previously reported values for the inducible type ID β-lactam of *P. aeruginosa* (Table 2). In contrast, inconsistent hydrolysis of ceftazidime was measured at low substrate concentrations (0.5 to 10 μM; Fig. 1). The slow decreases in A_{257} seen in some assays were apparently unrelated to the substrate concentration used. Since the change in absorbance at low substrate concentrations upon complete hydrolysis with base was minimal (e.g., 3 μM ceftazidime yielded 0.02 absorbance unit), this precluded a study at lower concentrations. Thus, we were unable to directly demonstrate ceftazidime hydrolysis by the periplasmic β-lactamase of the resistant variant (PA-48) or induced parental strain (PA-96).

In contradistinction, ceftazidime acted as an efficient competitive inhibitor of nitrocefin hydrolysis by periplasmic β-lactamase of variant strain PA-48 (Fig. 2). A K_i value of 11.4 ± 1.3 μM was obtained, in agreement with literature values for inducible, type Id chromosomal β-lactamases shared by all *P. aeruginosa* strains (Table 2). The properties of the induced β-lactamase from the parent strain (PA-96) and the constitutive β-lactamase from the resistant variant PA-60 were not as exhaustively studied; however, the similar K_m values for nitrocefin and cephaloridine and identical molecular weights for these enzymes (data not shown) suggested that they too were typical type Id chromosomal β-lactamases of *P. aeruginosa* (6).

β-Lactamase characterizations. The salient difference between the complement of periplasmic proteins in the two ceftazidime-resistant variants and the parental strain was the presence of a major band at ~41,000 molecular weight in the variants but not in the uninduced parent (data not shown). Of note, this band became prominent in the parental strain after exposure to a sub-MIC of ceftazidime (4 μg/ml) (data not shown). It was confirmed by isoelectric focusing and two-dimensional electrophoresis that the β-lactamase activity with a pI of 8.0 had an apparent molecular weight of ~40,000, in close agreement with molecular weight data from one-dimensional SDS-PAGE. Moreover, the results of the molecular weight and pI studies are in close agreement with those previously published for typical type Id chromosomal β-lactamases of *P. aeruginosa* (3, 20) (Table 2).

Modification of bioactivity. The parent strain and the two

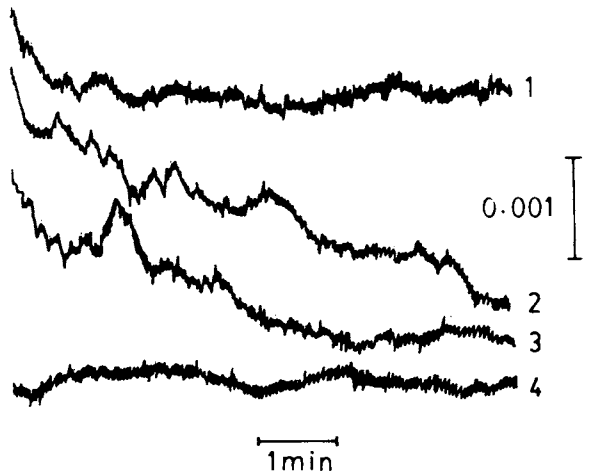

FIG. 1. Typical tracings of A_{257} as a function of time for the hydrolysis of 3.25 μM ceftazidime by extracted periplasmic β-lactamases of PA-48 (tracings 1, 2, and 3). Tracing 4 shows the result without enzyme included. Units on the *y* axis are A_{257} units. On the *x* axis, the bar indicates time in minutes. The left side of the tracing represents time zero, and the right side represents ~6.7 min. The tracings are stacked to allow them each to be shown without overlapping.

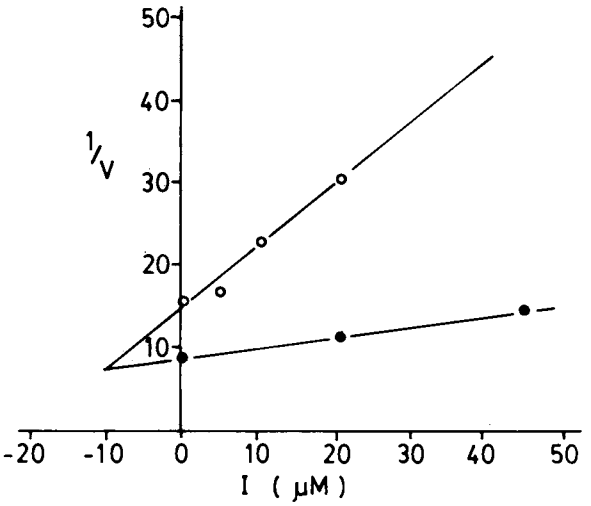

FIG. 2. Dixon plot of the inhibition, by different concentrations of ceftazidime, of nitrocefin hydrolysis. The reciprocal of the rate of hydrolysis (1/V in micromoles of nitrocefin hydrolyzed per milligram of protein per minute) was plotted against the inhibitor (I) concentration for two different concentrations of nitrocefin, 20.5 μM (○) and 40.9 μM (●). The lines intercept to the left of the *y* axis above the *x* axis, indicating competitive inhibition. The value of $-K_i$ is given by the *x* value equivalent to the intercept point.

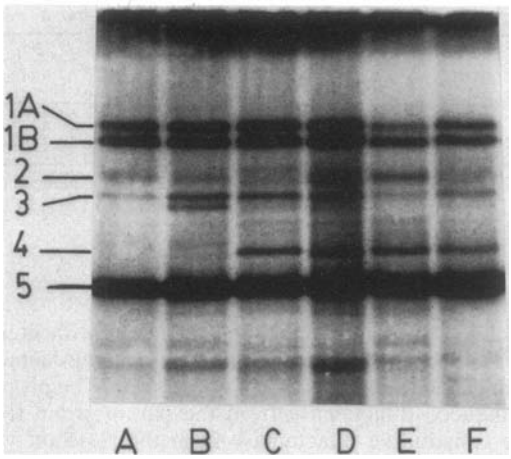

FIG. 3. Analysis of the PBPs of the parent strain PA-96 (lane F) and the ceftazidime-resistant isolates PA-48 (lane A) and PA-60 (lane B). For comparisons, three other ceftazidime-susceptible derivatives of PA-96 that had been passaged through the bacterial endocarditis animal model are shown (lanes C through E).

ceftazidime-resistant variants had zones of inhibition on the *E. coli* lawn virtually identical to that seen for the antibiotic disk alone (>20 mm), indicating no ceftazidime biomodification.

Outer cell envelope characterizations. No differences were observed in the one-dimensional SDS-PAGE profiles of the lipopolysaccharides or outer membrane proteins of the parental strain (PA-96) and the two resistant variants (data not shown). The amounts of individual major outer membrane proteins, including porin protein F, were quite similar in the three strains.

The outer membrane permeability of PA-96 yielded a permeability coefficient of 1.5×10^{-3} min per mg of cells per ml, a value similar to that obtained for our standard laboratory pseudomonal strain (PAO1) under similar conditions. PA-48 gave a permeability coefficient of 3.5×10^{-3} min per mg of cells per ml, indicating a slight, but not significant, increase in outer membrane permeability versus the reference strain.

Inner cell membrane characterization: PBPs. Strains PA-48 and PA-60 demonstrated one major PBP difference as compared with the parental strain (PA-96). In both resistant variants, PBP 4 was apparently missing, although a new band, running with lower relative mobility than PBP 4, was evident in the PBP profile of strain PA-60. This apparent loss of PBP 4 was not merely a consequence of passage through the rabbit endocarditis model; five randomly chosen pseudomonal isolates from cardiac vegetations of animals receiving amikacin monotherapy (three of which are shown in Fig. 3) had normal levels of PBP 4.

DISCUSSION

Recent experiences in our laboratory and others have clearly demonstrated that in vivo development of β-lactam resistances is an important determinant of therapeutic efficacy in invasive pseudomonal infections (2, 8, 28–30). However, the exact mechanisms of resistance have not been delineated in most of the clinically derived strains from these reports. The current study investigated possible mechanisms involved in the in vivo development of ceftazidime resistance in two pseudomonal variants, in direct comparison to the ceftazidime-susceptible, parental strain. The ceftazidime-resistant variants were stably derepressed for a β-lactamase that was normally inducible in the parental strain. The stable derepression, molecular weights and pI profile of this β-lactamase, in addition to its K_ms for cephaloridine and nitrocefin and the K_i for ceftazidime, are characteristic of the type Id β-lactamase of *P. aeruginosa* (6, 20). Structure-function studies of the outer membrane of the two variants eliminated alterations in the lipopolysaccharide profile (7), changes in the porin or other major outer membrane proteins, or decreases in outer membrane permeability as the cause of ceftazidime resistance. Additionally, there was no evidence for bioinactivation of ceftazidime by the resistant variants, and only the absence of PBP 4, an enzyme with no major discernible function in *P. aeruginosa*, was different when the PBP profiles of the resistant variants were compared with that of the parental strain.

P. aeruginosa has been shown to possess six PBPs designated 1A, 1B, 2, 3, 4, and 5 (24). Only one of these, PBP 5, has been definitely ascribed a function, as a D-alanine carboxypeptidase (23). However, on the basis of affinity for different β-lactams, binding kinetics and thermosensitivity, it has been suggested that PBPs 1A and 1B of *P. aeruginosa* correspond to 1B and 1A, respectively, of *E. coli* (15); PBPs 2, 3, and 4 in *P. aeruginosa* are structurally and functionally equivalent to those in *E. coli*. There apparently is no major PBP in *P. aeruginosa* corresponding to PBP 6 of *E. coli* (24). Assuming that this comparison is accurate and appropriate, it should be noted that the loss of PBP 4 in the current ceftazidime-resistant *P. aeruginosa* variants would have no major consequences. In *E. coli* cells, the carboxypeptidase function of PBP 4 is complemented by at least one other carboxypeptidase function provided by PBP 5 or 6 (33). For these reasons, it is difficult to envision PBP 4 as a primary target for ceftazidime in *P. aeruginosa*, and consequently its mutational loss should not result in ceftazidime resistance. Moreover, the studies of Hayes and Orr demonstrated that ceftazidime has substantially higher affinity for PBPs 3 and 1A of susceptible *P. aeruginosa* strains than for PBP 4 (11). PBPs 3 and 1A were apparently unaltered in the ceftazidime-resistant variants we examined.

It appears most likely that the ceftazidime resistance in variants PA-48 and PA-60 was related to the derepression of chromosomal type Id β-lactamase. Such strains have been sporadically isolated from clinical specimens and from laboratory-derived mutants, although to our knowledge this is the first report where such strains were isolated from a clinically relevant, controlled animal model (8, 14, 18, 28, 30, 37). Two mechanisms have been described whereby derepression of β-lactamase gives rise to resistance to β-lactamase stable or nonhydrolyzable β-lactams (16, 19) such as ceftazidime. Then and Anghern and others have presented data in favor of a nonhydrolytic barrier mechanism, whereby high β-lactamase levels in derepressed strains would bind with high affinity to such β-lactams and trap them in the periplasm, abbrogating accessibility to target PBPs (34). Since this mechanism involves 1:1 stoichiometry, an important corollary in this model is that the synthetic rate of new binding sites (i.e., new β-lactamase molecules) must balance the rate of diffusion across the outer membrane and appearance in the periplasm of new β-lactam molecules. Vu and Nikaido have presented convincing data of an alternative model (35) in demonstrating that many β-lactamase-stable β-lactams are actually hydrolyzed with low K_m (i.e., high affinity) and low V_{max} (i.e., low maximal activity rate). One previous study reporting ceftazidime hydrolysis by a

type Id pseudomonal β-lactamase was inconclusive, in that the evidence for hydrolysis was the inhibition by ceftazidime of a second β-lactam marker; however, inhibition of β-lactamase-induced hydrolysis of the marker could have occurred via ceftazidime–β-lactamase binding as in our current study. We were unable to directly confirm ceftazidime hydrolysis at low substrate concentrations by the pseudomonal β-lactamases under the conditions identical to those used by Vu and Nikaido (35) for their *Enterobacter* β-lactamase with either ceftazidime or other cephalosporin substrates. One explanation for this could have been that the K_m of the enzyme for ceftazidime was at the lower end of the tested range of substrate concentrations (0.5 to 10 μM) and that higher substrate concentrations above the K_m resulted in substrate inhibition. The phenomenon of substrate inhibition has been previously observed for *E. cloacae* β-lactamases against a variety of cephalosporins (31, 35) and in our laboratory for *P. aeruginosa* β-lactamases of PA-48 and PA-60 (versus cephaloridine and nitrocefin substrates; data not shown).

Despite our inability to directly measure ceftazidime hydrolysis by pseudomonal β-lactamases in the current study, we have eliminated the nonhydrolytic barrier model as the primary explanation for ceftazidime resistance. At a concentration of 50 μg of ceftazidime per ml outside the cell (i.e., below the MIC for both PA-48 and PA-60), we have calculated the rate of appearance of new β-lactam molecules in the periplasm. With the measured outer membrane permeability of nitrocefin, Fick's law as expressed by Zimmerman and Rosselet (40), and the assumption that the rate of ceftazidime uptake will be similar to nitrocefin, 10^3 molecules of ceftazidime will be taken up per resistant pseudomonal cell per s. The chromosomal β-lactamase in the resistant strains constitutes ~1% of the total cell protein (35), which corresponds to ~10^5 molecules per cell. Thus the enzymes molecules, to hydrolyze the incoming ceftazidime molecules, must have a turnover rate (molecules of ceftazidime hydrolyzed per molecule of enzyme per second) of 10^{-2}. The turnover rate of the purified type Id β-lactamase of *P. aeruginosa* (versus cephaloridine) has been experimentally determined to be 3,000 (20, 39). Thus, even a catalytic rate for ceftazidime that was 5 orders of magnitude lower than that measured for cephaloridine might account for resistance to 50 μg of ceftazidime per ml. Such a rate would not be measurable with currently available techniques. The nonhydrolytic trapping mechanism would not be sufficient to explain ceftazidime resistance in our strains. The 10^5 molecules of β-lactamase would become saturated (bound) by periplasmic ceftazidime molecules in only 100 s; this would require the cell to synthesize 10^3 molecules of β-lactamase per s to allow trapping of ceftazidime molecules subsequently diffusing into the periplasm (2.4×10^6 molecules per cell with the generation time of 40 min). However, the calculated rate of β-lactamase synthesis in our derepressed mutants is only 4×10^1 enzyme molecules per s per cell. We therefore postulate that slow ceftazidime hydrolysis, rather than nonhydrolytic β-lactamase trapping, must be the principal mechanism of resistance in these *P. aeruginosa* strains.

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