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Evidence for Multiple Forms of Type I Chromosomal β-Lactamase in *Pseudomonas aeruginosa*

MARK L. GATES, CHRISTINE C. SANDERS,* RICHARD V. GOERING, AND W. EUGENE SANDERS, JR.
Department of Medical Microbiology, Creighton University School of Medicine, Omaha, Nebraska 68178

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The multiple stages of derepression of the type I chromosomal beta-lactamase in *Pseudomonas aeruginosa* were examined. Mutants partially and fully derepressed for beta-lactamase were selected from a wild-type clinical isolate. An analysis of the beta-lactamase produced by these mutants and the induced wild type revealed significant differences in the products of derepression at each stage. Beta-lactamase produced by the fully derepressed mutant showed a lower affinity (K_m, 0.113 mM) for cephalothin than that produced by the partially derepressed mutant (K_m, 0.049 mM). However, due to a very large V_{max}, the former possessed a much greater hydrolytic efficiency. Differences in substrate profile were also noted. Only beta-lactamase from the fully derepressed mutant hydrolyzed cefamandole, cefoperazone, and cefonicid. The partially derepressed mutant possessed a single beta-lactamase band with a pI of 8.4. The fully derepressed mutant possessed this band and an additional major band with a pI of 7.5. Induction of the wild type with cefoxitin produced both bands. The changes in physiologic parameters of the enzymes produced in the different stages of derepression suggest a complex system for beta-lactamase expression in *P. aeruginosa*. This may involve at least two distinct structural regions, each of which is under control of the same repressor.

Pseudomonas aeruginosa characteristically possesses a chromosomally mediated beta-lactamase (6, 9, 21). This enzyme, primarily a cephalosporinase, belongs to the Richmond and Sykes type I beta-lactamases, and is often referred to as the Sabath and Abraham enzyme after the investigators who first described it (22, 23). Unlike many plasmid-mediated beta-lactamases, this enzyme is not produced constitutively but is inducible. Induction (derepression) of this beta-lactamase results in a multiple beta-lactam resistance, which involves not only the cephalosporins but also the antipseudomonal penicillins and monobactams (11, 12, 15, 24).

P. aeruginosa appears to undergo derepression of its beta-lactamase at several levels (1, 2, 5, 8, 12, 16, 20, 25, 27). Most isolates reported to date are only partially derepressed for beta-lactamase. In these isolates, base-line levels are significantly elevated over those usually found in wild-type *P. aeruginosa*, but levels can be increased even further by use of an enzyme inducer. There have been only a few isolates described in which the beta-lactamase is fully derepressed. These appear to produce high levels of enzyme constitutively. To date there has been no systematic evaluation of derepression of beta-lactamase in *P. aeruginosa*. Thus, the current study was designed to evaluate the different stages of derepression of beta-lactamase in *P. aeruginosa* and analyze the product of each stage.

MATERIALS AND METHODS

Bacterial isolates. The pretreatment (no. 164) isolate of *P. aeruginosa* recovered from patient 4 described by Preheim et al. (20) was evaluated in the present study. This isolate was serotype 2 and did not possess a plasmid-mediated beta-lactamase. Two mutants from isolate no. 164 were selected in the laboratory. Mutant 164M1 was selected from no. 164 by a single passage in broth containing 32 μg of cefotaxime per ml. Mutant 164CD was selected from no. 164 by a single passage in agar containing 64 μg of ceftazidime per ml. All mutants were highly stable and could be maintained on drug-free media.

Antibiotics. Working antibiotic standards were prepared on the day of use from the following powders: azlocillin sodium, mezlocillin sodium (Miles Pharmaceuticals, West Haven, Conn.), imipenem, cefoxitin sodium (Merck, Sharp and Dohme, West Point, Pa.), cloxacillin sodium (Sigma Chemical Co., St. Louis, Mo.), tetracycline hydrochloride, BMY-28142 sulfate, amikacin base, ceforanide lysine (Bristol Laboratories, Syracuse, N.Y.), ceftazidime pentahydrate, nitrocephin (Glaxo Ltd., Middlesex, England), cefamandole lithium, cephalothin sodium (Eli Lilly & Co., Indianapolis, Ind.), potassium clavulanate (Beecham Laboratories, Bristol, Tenn.), cefotaxime sodium, HR810 sulfate (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.), chloramphenicol (Parke Davis & Co., Detroit, Mich.), piperacillin (Lederle Laboratories, Pearl River, N.Y.), cefoperazone sodium, cefonicid sodium (Roerig-Pfizer, New York, N.Y.), ceftriaxone disodium (Hoffmann-LaRoche Inc., Nutley, N.J.), and gentamicin sulfate (Schering Corp., Bloomfield, N.J.).

Susceptibility tests. MICs of all antibiotics were determined in Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.) in a final volume of 3 ml with an inoculum of 10^5 CFU/ml. The MIC was defined as the lowest concentration preventing growth after incubation at 37°C in air for 24 h. Differences fourfold or greater were considered significant.

Beta-lactamase assays. Beta-lactamase activity was determined in spectrophotometric assays with 100 μM concentrations of each substrate (7). Assays with whole cells entailed a 4-h incubation period at 37°C. The cells were removed by microfiltration prior to assay. Beta-lactamase was induced by growing the isolates overnight on 100 μg of cefoxitin per ml in Mueller-Hinton agar prior to the 4-h incubation. In tests with sonic extracts, incubation lasted 5 to 10 min with results recorded at 1-min intervals. Beta-lactamase was induced by incubating the isolates for 2 to 6 h in Mueller-

* Corresponding author.

TABLE 1. Beta-lactamase activity in *P. aeruginosa* 164 and its mutants

Isolate	Enzyme status	Beta-lactamase activity						
		Whole cells[a]		Sonic extract[b]				
		Cephalothin	Cefamandole	Cephalothin	Ceforanide	Cefoperazone	Cefamandole	Cefonicid
164	Uninduced	21 ± 4	3 ± 1	—[c]	—	—	—	—
	Induced	87 ± 3	66 ± 4	163 ± 54	34 ± 8	—	—	—
164Ml	Uninduced	89 ± 3	10 ± 3	24 ± 9	9 ± 2	—	—	—
	Induced	91 ± 2	69 ± 5	502 ± 149	106 ± 22	6 ± 2	—	—
164CD	Uninduced	90 ± 3	83 ± 5	1,453 ± 193	366 ± 76	16 ± 4	11 ± 2	5 ± 1
	Induced	89 ± 3	82 ± 4	1,521 ± 286	427 ± 36	20 ± 2	13 ± 2	6 ± 1

[a] Nanomoles of substrate hydrolyzed per 10^9 cells. Mean ± standard deviation calculated from five cell preparations. Induction was performed by growing the strains overnight on Mueller-Hinton agar with 100 μg of cefoxitin per ml.

[b] Nanomoles of substrate hydrolyzed per minute per milligram of protein. Mean ± standard deviation calculated from three to four enzyme preparations. Induction was continued for 2 h.

[c] —, Activity below accurately detectable level.

Hinton broth containing subinhibitory concentrations of inducer. After this induction period, 1 mM 8-hydroxyquinoline was added to arrest protein synthesis. The cells were harvested and washed twice, and sonic extracts were prepared in 0.1 M phosphate buffer, pH 7.0.

Kinetic parameters were established for the beta-lactamase activity in sonic extracts of isolates 164M1 and 164CD with cephalothin as a substrate and cloxacillin as an inhibitor. K_m and V_{max} were determined from Lineweaver-Burk plots for various substrate concentrations. K_i was determined from a secondary repeated plot of the slopes of Lineweaver-Burk plots for various cloxacillin concentrations (4).

Isoelectric focusing. Beta-lactamases were focused in 7% polyacrylamide (165 by 215 by 2 mm) gels containing pH 3 to 10 ampholytes by the method of Vecoli et al. (26). The gels were focused across the width at 4°C, 1,600 V, for 90 min with an LKB Multiphor unit (LKB Instruments Inc., Rockville, Md.). Because of difficulties in detecting minor bands on the focused gel with nitrocephin-soaked filter paper, the detection system was modified. The focused gel was overlaid with molten agar containing 50 μg of nitrocephin per ml. This overlay procedure diminished diffusion of the major bands while minor bands were developing and allowed pictures to be taken of the gel for up to 1 h after application of the nitrocephin agar. The developed gel was photographed with a Polaroid MP-4 camera equipped with a Tiffen 58 dark green filter and type 51 high-contrast film. For inhibitor studies, filter paper soaked with the desired inhibitor was placed on the surface of the focused gel. After 10 s, the paper was removed, and the nitrocephin overlay was applied.

RESULTS

Beta-lactamase activity. The beta-lactamase activity of *P. aeruginosa* 164 and its mutants was initially assessed in whole-cell assays. *P. aeruginosa* 164 possessed activity that was inducible by cefoxitin when both cephalothin and cefamandole were used as substrates (Table 1). Mutant 164M1 appeared to be fully derepressed when tested against cephalothin but still possessed cefoxitin-inducible activity when tested against cefamandole. Mutant 164CD was fully derepressed for beta-lactamase activity against both cephalothin and cefamandole.

Since substrate exhaustion in whole-cell assays limited the examination of the inducibility of beta-lactamase when cephalothin was used as the substrate, tests were also performed on sonic extracts. Isolate 164 and mutant 164M1 possessed inducible activity against cephalothin, while mutant 164CD appeared to be fully derepressed (Table 1). Uninduced (base line) beta-lactamase activity detected with cephalothin as a substrate was 24 times higher in 164M1 than in 164 and 61 times higher in 164CD than in 164M1. Similar results were obtained when ceforanide was used as a substrate (Table 1). When cefoperazone, cefamandole, and cefonicid were used as substrates, no activity could be detected in uninduced sonic extracts of 164 or 164M1. Activity in sonic extracts of 164CD appeared to be constitutive. These observations further suggested that there was a progression of derepression in *P. aeruginosa* 164.

Mutation frequencies. Mutation frequencies for 164M1 and 164CD, two mutants selected directly from *P. aeruginosa* 164, were determined by inoculating agar containing superinhibitory concentrations of ceftazidime or cefotaxime with 10^7, 10^8, or 10^9 CFU of *P. aeruginosa* 164. Mutation frequencies of 10^{-7} and 10^{-9} were obtained for 164M1 and 164CD.

Antibiotic susceptibility. The antibiotic susceptibility of *P. aeruginosa* 164 and its mutants was determined in broth dilution tests. *P. aeruginosa* 164M1, the mutant partially derepressed for beta-lactamase, was highly resistant to all antipseudomonal penicillins and cephalosporins, except BMY-28142 (Table 2). The fully derepressed mutant 164CD

TABLE 2. Antibiotic susceptibility of *P. aeruginosa* 164 and its mutants

Antibiotic	MIC (μg/ml) for *P. aeruginosa* isolate:		
	164	164Ml	164CD
Cefotaxime	32	>1,024	>1,024
Ceftriaxone	128	>1,024	>1,014
Cefoperazone	128	1,024	1,024
Ceftazidime	4	128	256
HR 810	16	256	256
BMY-28142	8	32	128
Mezlocillin	128	>1,024	>1,024
Piperacillin	16	>1,024	>1,024
Carbenicillin	128	1,024	1,024
Imipenem	4	4	8
Gentamicin	4	4	4
Amikacin	8	8	16
Tetracycline	32	32	32
Chloramphenicol	128	128	128

was highly resistant to all antipseudomonal penicillins and cephalosporins, including BMY-28142. Susceptibility to imipenem, aminoglycosides, tetracycline, and chloramphenicol was the same for the mutants as for *P. aeruginosa* 164.

Analysis of the products of derepression. There are two possible explanations for the differences between partially and fully derepressed mutants in their ability to hydrolyze various substrates. First, the higher amounts of enzyme found in the fully derepressed mutant may have been necessary to detect hydrolysis of cefoperazone, cefamandole, and cefonicid, substrates less labile than cephalothin. Secondly, the fully derepressed mutants may possess an altered enzyme activity not detectable in the wild-type or partially derepressed mutants without induction. This activity may be responsible for the hydrolysis of the additional substrates. To assess these possibilities, a sonic extract from mutant 164CD was diluted to yield the same beta-lactamase activity (per unit volume) against cephalothin as sonic extracts from 164M1. These diluted sonic extracts from 164CD did not hydrolyze cefamandole. Sonic extracts from 164M1 were then concentrated to yield the same beta-lactamase activity (per unit volume) against cephalothin as sonic extracts from 164CD. However, these concentrated sonic extracts could not be used in the spectrophotometric assay, for they were deep red. Hence, an indirect assay was developed. The concentrated sonic extract from 164M1 was incubated with 210 μM cefamandole. Every 15 min, a portion was removed, diluted, and added to phosphate buffer containing cephalothin so as to give final concentrations of 10 μM cefamandole and 100 μM cephalothin. In this assay, cefamandole, if not previously hydrolyzed by the concentrated sonic extract, would function as an inhibitor of cephalothin hydrolysis. However, if hydrolysis of cefamandole occurred, the inhibition of cephalothin hydrolysis would decrease with time. Under these conditions, the sonic extract from strain 164CD hydrolyzed the cefamandole. In controls, 10 μM cefamandole inhibited cephalothin hydrolysis by 60% when the cefamandole was added after the concentrated 164M1 sonic extract had been diluted for assay against cephalothin. In the test, hydrolysis of cephalothin was inhibited by 70% when cefamandole was incubated with the concentrated 164M1 sonic extract for 15, 30, 45, 60, and 75 min prior to dilution for assay. Thus, there appeared to be no hydrolysis of cefamandole by the concentrated 164M1 sonic extract. These results suggested that hydrolysis of cefamandole seen only after induction of *P. aeruginosa* 164 or its partially derepressed mutant, and without induction in the fully derepressed mutant, was due to an altered enzyme activity.

The K_m, V_{max}, and K_i were determined for the beta-lactamase activity in sonic extracts prepared from *P. aeruginosa* 164M1 and 164CD. These kinetic parameters could not be determined for *P. aeruginosa* 164 because there was insufficient activity in uninduced sonic extracts. The 164M1 beta-lactamase had a much higher affinity for cephalothin (low K_m) than the 164CD beta-lactamase ($P < 0.05$) (Table 3). However, the V_{max} for 164CD was 76 times higher than for 164M1 ($P < 0.05$). Thus, the hydrolytic efficiency of the 164CD enzyme for cephalothin was almost 33 times greater than that of the 164M1 enzyme. Susceptibility of the 164CD enzyme to inhibition by cloxacillin was 10 times less than that of the 164M1 enzyme.

TABLE 3. Kinetic parameters of beta-lactamase activity[a]

Enzyme prepn	V_{max} (nm/min per mg of protein)	K_m (mM)	V_{max}/K_m	K_i (M)
164M1	0.147 ± 0.017[b]	0.049 ± 0.005	3	1.6×10^{-9}
164CD	11.194 ± 1.0	0.113 ± 0.01	100	1.5×10^{-8}

[a] V_{max} and K_m determined with cephalothin as a substrate. K_i determined with cloxacillin.

[b] Mean from seven different enzyme preparations ± standard deviation.

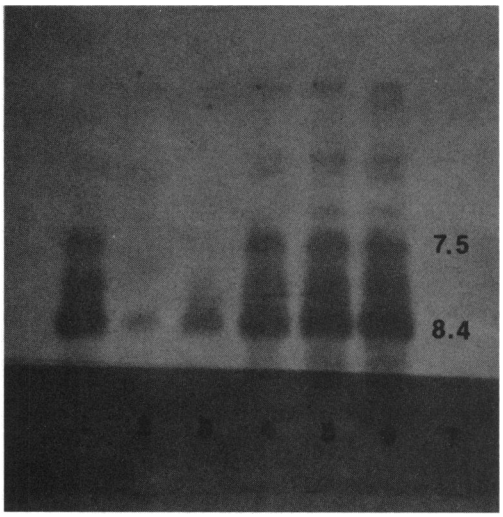

FIG. 1. Isoelectric focusing of beta-lactamases from *P. aeruginosa* 164 (lanes 1 and 2), 164M1 (lanes 3 and 4), and 164CD (lanes 5 and 6). A 10-μl volume of each enzyme was applied. Lanes 2, 4, and 5 contain enzyme preparations from uninduced cultures, while lanes 1, 3, and 6 contain enzyme preparations from cultures induced for 2 h with 100 μg of cefoxitin per ml.

Isoelectric focusing. Initial attempts to focus the beta-lactamases present in *P. aeruginosa* 164 and its mutants failed to detect any additional bands in 164CD or in 164 and 164M1 after cefoxitin induction. A single major band with a pI of 8.4 was detected in sonic extracts from all isolates. In these initial tests, samples from each sonic extract containing an equivalent amount of total enzyme activity (as determined with nitrocephin) were being applied to the gel (26). This necessitated significant dilution of the sonic extracts, especially those prepared from 164CD. Since previous tests had shown that the cefamandole hydrolyzing activity in sonic extracts from 164CD could be easily diluted out, each sonic extract was applied to the gel without dilution. In these undiluted sonic extracts, a major band with a pI of 8.4 was still detected in all sonic extracts (Fig. 1). However, an additional major band with a pI of 7.5 was detected in the sonic extracts prepared from 164CD (Fig. 1, lane 5). This band was not observed in sonic extracts prepared from *P. aeruginosa* 164 or 164M1 (Fig. 1, lanes 2 and 3). It appeared in sonic extracts prepared from these isolates after induction for 2 h with cefoxitin (Fig. 1, lanes 1 and 4).

All of the beta-lactamase activity in each band could be inhibited by applying a filter paper soaked with 0.1 mM cloxacillin prior to the nitrocephin overlay. No diminution in any band was observed when 0.1 mM potassium clavulanate was used as an inhibitor.

DISCUSSION

The results of this study indicate that there are at least two stages for sequential derepression of type I chromosomal beta-lactamase in *P. aeruginosa*. The first stage was characterized by a partial derepression of beta-lactamase activity represented by mutant 164M1. This mutant possessed in-

creased base-line levels of beta-lactamase over that of *P. aeruginosa* 164 when cephalothin was used as the substrate. However, higher levels of enzyme activity against cephalothin and ceforanide could still be induced by exposure to cefoxitin. The second stage of derepression was characterized by mutant 164CD. This strain possessed high levels of beta-lactamase activity against cephalothin and ceforanide and moderate activity against cefamandole, cefoperazone, and cefonicid. None of these activities could be increased by exposure to cefoxitin.

Although mutants of *P. aeruginosa* that are partially or fully derepressed for type I beta-lactamase have been reported previously (1, 2, 5, 8, 12, 16, 20, 25, 27), this study was the first examination of the sequential derepression of beta-lactamase in the same strain. The major finding of this study was the large difference between the beta-lactamase preparations of the partially and fully derepressed mutants. The significant differences in K_m, V_{max}, K_i, and substrate profile of the beta-lactamases from mutants 164M1 and 164CD suggest the presence of multiple distinct forms of the type I enzyme in *P. aeruginosa*. The precise relationship between the additional band with a pI of 7.5 and the altered enzyme kinetics and substrate profile will require further study. It may merely reflect a satellite band often encountered when larger quantities of enzyme are focused, or it may in fact represent new enzyme. Purification will be required to assess these possibilities. However, a change in pI does not always accompany changes in substrate profile (13). Thus, the altered kinetics and substrate profile of the beta-lactamase in 164CD with or without an altered pI support the presence of multiple forms of type I beta-lactamase in *P. aeruginosa*.

A comparison of the parameters in the literature also suggests a broad heterogeneity in this enzyme in *P. aeruginosa* (1, 2, 5, 6, 9, 14, 17–19, 21–23). The K_m for cephalothin ranges from 0.04 to 0.7 mM. The K_i for cloxacillin varies from 10^{-7} to 10^{-9} M, while pIs vary from 7.2 to 8.7. Although this heterogeneity probably reflects strain variation as well as differences in source and purity of enzyme, the results of our study suggest a similar heterogeneity within a single strain when tested under identical conditions. Thus, it appears that derepression of type I beta-lactamase in *P. aeruginosa* may give rise to multiple distinct forms of the enzyme. All forms are type I enzymes because of their cephalosporinase activity, alkaline pIs, high susceptibility to inhibition by cloxacillin, and resistance to potassium clavulanate.

The genetic relationship between the products of partial and full derepression is unclear. King et al. have suggested that partial derepression results from a mutation in the operator gene, while full derepression results from a mutation in the repressor protein (12). However, were this the case, one should not detect any differences in the products of derepression between the two stages. A somewhat different explanation is supported by the additional data provided by this study. The mutation frequency for partial derepression (10^{-7}) was lower than that for full derepression (10^{-9}), suggesting that both stages of derepression could be caused by separate mutational events in closely related (or even the same) genes. Perhaps there are multiple structural gene sequences, each under negative control by the same repressor. The promoter regions for the two sequences may possess different affinities for the same repressor. Thus, a single mutational event affecting the repressor would lead to initial loss of repressor binding to the promoter region with the weakest affinity, i.e., partial-derepression phenotype. A second mutational event would be required to alter the repressor sufficiently so that it could no longer bind to the promoter region of greatest affinity, i.e., full derepression. Additional support for the presence of at least two structural regions, each under negative control by the same repressor, has been provided by the work of Curtis et al., Livermore, and Jacobs et al. (3, 11, 15). These investigators have examined a series of *P. aeruginosa* mutants derived from the same strain. From the wild type a beta-lactamase-negative mutant was derived by mutagenesis. Two types of spontaneous revertants were recovered from this mutant. Both produced beta-lactamase constitutively. However, one revertant produced a high level of enzyme (6,343 U of activity), while the other produced only a low level of beta-lactamase (426 U of activity). The ease with which these revertants were obtained suggested that a separate mutational event in different structural regions may have been responsible for each. Direct examination of this possibility, however, will require an in-depth analysis of the beta-lactamases produced by each of these mutants. The genetic map recently published for *P. aeruginosa* PAO also supports a complex genetic system for the type I beta-lactamase in this organism (10). This map shows two *bla* regions, one at 37 min and the other at 55 to 60 min. Perhaps these represent the two distinct regions that possess both control and structural genes for the pseudomonas type I enzyme. Clearly, more detailed analysis of the genes as well as the gene products will be needed before this complex system is fully understood.

Despite the complexity of the genetic apparatus controlling beta-lactamase expression in *P. aeruginosa*, it is interesting to note that partial derepression is sufficient to confer resistance to most of the antipseudomonal beta-lactams. This is probably why most *P. aeruginosa* mutants isolated from patients treated with these drugs are only partially derepressed. It is tempting to speculate that the development of even more potent antipseudomonal beta-lactams will encourage a greater occurrence of fully derepressed mutants. This possibility will require close scrutiny as newer drugs are developed and evaluated as potential chemotherapeutic agents for infections due to *P. aeruginosa*.

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