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Effect of pH and CO_2 on *In Vitro* Susceptibility of *Pseudomonas cepacia* to β-Lactams

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ABSTRACT. Inhibition of *Pseudomonas cepacia* (but not *Pseudomonas aeruginosa*) by β-lactams was decreased in 5% CO_2 in air compared with air alone. The effect of CO_2 and pH (range, 6.0 to 8.0) on β-lactam susceptibility, β-lactamase expression, and outer membrane proteins was studied in isolates recovered from the sputum of children with cystic fibrosis. Incubation in 5% CO_2 decreased the activity of piperacillin, piperacillin/tazobactam, and ceftazidime, although isolates were still clinically sensitive (minimum inhibitory concentrations < 16 mg/L). Cefpirome activity was markedly decreased from a minimum inhibitory concentration of 2.0 to greater than 64 mg/L. On highly buffered 3-(*N*-morpholino)-propane sulfonic acid media, β-lactam susceptibility was eliminated at pH greater 7.5. A 2- to 13-fold increase in β-lactamase activity was demonstrated after growth in 5% CO_2 compared with basal aerobic levels for 13 of 15 clinical isolates. β-Lactamase activity did not vary significantly with pH. Addition of imipenem to media (2.0 mg/L) resulted in hyperproduction of β-lactamase (180-fold). Isoelectric points varied with cultural conditions, and all β-lactamases detected were inhibited by clavulanate and tazobactam. Significant hydrolysis of piperacillin and ceftazidime could not be demonstrated. A 36-kD porin was present at all pH tested. Thus, our strains of *Pseudomonas cepacia* were markedly affected by cultural conditions not normally used in standardized susceptibility tests. However, such conditions may be encountered in the pathologically altered infected lung in cystic fibrosis. (*Pediatr Res* 35: 299–302, 1994)

Abbreviations

OMP, outer membrane protein
MIC, minimum inhibitory concentration
MOPS, 3-(*N*-morpholino)-propane sulfonic acid
HBMM, highly buffered MOPS media
I_{50}, concentration of inhibitor required to inhibit enzymatic activity by 50% under defined assay conditions

Pseudomonas cepacia has emerged as an important opportunist pathogen in pulmonary infections in children with cystic fibrosis (1–3) and its appearance is associated with increased morbidity and mortality (2). Many strains of *P. cepacia* are resistant to many antimicrobial classes, including β-lactams, polymyxins, aminoglycosides, and quinolones. With some β-lactams, even when *in vitro* activity was demonstrable, it was not translated into clinical efficacy (3). Lack of *in vivo* activity in this difficult-to-treat group of patients has been attributed to a variety of factors, including reduced permeation of antibiotic across bronchial mucosa, poor perfusion into all areas of the lung, high infecting inocula (>10^7 bacteria/mL), impaired phagocytosis, and antibiotic inactivation by pus (3).

The possibility that this particular microorganism may respond differently to β-lactams *in vivo*, compared with that demonstrated in the artificial environment of *in vitro* laboratory susceptibility tests, has not been investigated previously. We report the effect of alterations in CO_2 concentration and pH on the susceptibility of *P. cepacia* to β-lactams and their effect on bacterial β-lactamases and outer membrane proteins.

MATERIALS AND METHODS

Test strains. Fifteen isolates of *P. cepacia* recovered from the sputum of 15 children admitted to Alder Hey Children's Hospital with cystic fibrosis were studied. All isolates were identified by growth at 30°C with a commercial identification kit (API, Basingstoke, UK). Detailed findings are presented only for *P. cepacia* (laboratory code PC7) as our typical isolate. *P. aeruginosa* NCTC 10662 was used to control susceptibility testing. *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as controls to determine β-lactam hydrolysis profiles (4). β-Lactamases of known pI were obtained from a laboratory strain of *E. coli* RLUH 20 (pI 5.4) and *P. paucimobilis* (pI 4.6) (5).

Susceptibility testing. Resistance phenotypes were determined with a controlled disc diffusion method and MIC by the "E" test method (AB Biodisk, Solna, Sweden) (6). Susceptibilities were performed on both Iso-Sensitest agar (Oxoid Ltd., Basingstoke, UK) and a minimal growth medium (7) buffered with 0.1 M MOPS to pH 6.0, 6.5, 7.0, 7.5, and 8.0. This HBMM permits a reliable maintenance of pH during incubation. Plates were incubated overnight at 37°C in air and in 5% CO_2 in air. Preincubation and postincubation pH of agar from uninoculated areas and areas scraped free of bacterial growth were determined with a surface-reading electrode.

β-Lactamase extraction. Overnight bacterial growth was suspended from test media in 0.1 M potassium phosphate buffer, pH 7.0. Sonication was performed for up to 5 × 30 s pulses with samples immersed in an ice-water slurry. Cell-free sonicates were prepared by centrifugation at 13 000 rpm for 10 min and sterilized by filtration through a 0.22-μm disposable filter (Millipore Ltd., Molsheim, France). Protein concentrations was determined by the method of Lowry *et al.* (8). Supernatants were stored at −70°C. β-Lactamase activity was determined for isolates grown in air, in 5% CO_2 in air, and in the presence of imipenem (2.0

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mg/L) as an inducing agent using 100 μM nitrocefin (Oxoid Ltd.) as substrate. Specific enzyme activities were measured at 482 nm (9), and activity of the enzyme was defined as micromoles of nitrocefin destroyed per milligram of protein. Substrate profiles were performed by the disc inactivation method (4) with 20 μL of sonicate (adjusted to 1 mg protein/mL) per disc. The I_{50} of the β-lactamase inhibitors clavulanic acid (SmithKline Beecham, Brentford, UK) and tazobactam (Lederle, Pearl River, NY) were determined against basal level and induced β-lactamases (10) with nitrocefin as substrate.

pI determination. Isoelectric focusing was performed with cell-free sonicates applied to polyacrylamide gels (pH range, 3.0–9.5). After electrophoresis, β-lactamase bands were visualized by placing a filter paper soaked in a 100 μM solution of nitrocefin on the gel. The pH gradient was calibrated with proteins of known pI (LKB, Pharmacia, Uppsala, Sweden).

Characterization of outer membrane proteins. P. cepacia was grown at 37°C in Luria broth (Difco Ltd., Detroit, MI) (pH range, 6.0–8.0) to an absorbance of approximately 0.8 OD units at 450 nm. Bacterial membranes (inner and outer) were obtained by centrifuging sonicated lysates at 100 000 × *g* for 1 h at 4°C, and outer membrane fractions were obtained by centrifugation after incubation in *N*-lauryl sarkosinate (0.5%). SDS-PAGE was carried out (11) with a resolving gel composition of 9% wt/vol acrylamide (36:0.84) with 4 M urea and a stacking gel of 4.5% wt/vol acrylamide (18:0.84) (12). Proteins were visualized with Coomassie blue.

RESULTS

At 37°C in air, all isolates appeared susceptible to piperacillin, piperacillin/tazobactam, cefpirome, and ceftazidime but varied from moderately susceptible to resistant to aztreonam, temocillin, and imipenem. Susceptibility to cefpirome and, when present, to aztreonam, temocillin, and imipenem was lost on incubation in 5% CO_2 in air. Piperacillin, piperacillin/tazobactam, and ceftazidime activity was decreased but not eliminated. This finding was not observed with *P. aeruginosa* NCTC 10662. With *P. cepacia* (laboratory code PC7) as a typical isolate, a decrease in susceptibility (MIC) to β-lactams when grown on HBMM was found with pH < 7.5 (Table 1). Similar increases in β-lactam MIC occurred at pH 7.0 for all *P. cepacia* isolates tested. Again, the susceptibility of the control strain of *P. aeruginosa* was not affected. Addition of imipenem to Iso-Sensitest agar (2.0 mg/L) antagonized all β-lactams. This is probably a result of the known ability of imipenem to induce β-lactamase expression.

Before incubation, Iso-Sensitest agar had a surface pH of 7.2, but after overnight incubation in air, uninoculated areas of the agar had a pH of 8.3 and beneath bacterial growth the pH was 9.05. In 5% CO_2 in air, uninoculated areas of the agar had a pH of 7.1, and beneath the bacterial growth pH was 8.2. This difference represents approximately 1 pH unit under areas of bacterial growth between the two incubation atmospheres. The pH of HBMM in clear and inoculated areas varied by only ± 0.2 pH units regardless of incubation atmospheres.

The effects of pH, incubation atmosphere, and presence of imipenem as an inducing agent on β-lactamase activity on isolate PC7 are summarized in Table 2. Incubation in CO_2 increased the aerobic basal level of β-lactamase production by 10-fold and the presence of imipenem by 180-fold. The β-lactamase activity of 12 of 14 other *P. cepacia* isolates was increased by 200–1300% by incubation in CO_2. For the remaining two isolates β-lactamase activity was increased by 50%. Incubation of PC7 in air produced a single β-lactamase band at pI 7.7. Incubation in 5% CO_2 in air induced two further bands of pI 6.5 and 8.2. On HBMM, expression of β-lactamase was pH dependent. With growth at pH 8.0, a band of β-lactamase was not detected at pI 7.9, and only a trace was detectable at pH 6.5. Three bands at pI of 6.5, 7.9, and 8.2 were detected with growth at pH < 7.0. Multiple, strongly staining bands of β-lactamase activity were detected in sonicates from imipenem-induced cultures. Specific enzyme activity (micromoles of nitrocefin hydrolyzed per milligram of protein) did not alter with pH. Regardless of cultural conditions, all β-lactamases were susceptible to both clavulanate and tazobactam inhibition as measured by I_{50} values.

With the disc inactivation test (Table 3), the basal level of aerobic β-lactamase from PC7 had minimal activity against the β-lactam substrates. Enzymes induced by growth in 5% CO_2 in air showed greater activity toward 1st- and 2nd-generation cephalosporins, including cefuroxime. Imipenem-induced β-lactamase showed extra activity toward the 3rd-generation cephalosporins, cefotaxime and ceftizoxime, and the monobactam, aztreonam.

Resistance to piperacillin, piperacillin/tazobactam, and ceftazidime correlated with cultural conditions that were associated with expression of a β-lactamase of pI 7.9. However, the methodologic procedure used did not demonstrate marked hydrolysis of either piperacillin or ceftazidime by sonicates of bacteria grown in the presence of imipenem or from strains grown on HBMM at pH < 7.5 (*i.e.* those containing β-lactamase of pI 7.9). SDS-PAGE of the outer membranes of PC7, grown over the pH range of 6.0 to 8.0, showed a single 36-kD porin with no discernible difference in bands or intensity in bacteria grown at each pH.

DISCUSSION

P. cepacia has emerged as an important pathogen associated with severe and often fatal infection in children and adults with cystic fibrosis (1, 2, 13). Institution of the most appropriate antimicrobial chemotherapy is of major importance in managing such infections (3, 14). Such therapy is often guided by *in vitro* tests of antimicrobial sensitivity. Because alterations in pH have been shown to alter apparent antimicrobial activity (15), to standardize interlaboratory testing such tests are performed in air (16, 17) on media with an initial pH of 7.4 ± 0.2. However,

Table 1. *Effect of pH and CO_2 on β-lactam susceptibility (MIC)* for P. cepacia (PC7) and P. aeruginosa NCTC 10662 (CONT.)*

	Ceftazidime				Piperacillin				Piperacillin plus tazobactam				Cefpirome			
	PC7		CONT.		PC7		CONT.		PC7		CONT.		PC7		CONT.	
Test media	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
HBMM																
pH 8.0	0.75	3.0	1.0	1.0	3.0	12	1.5	1.5	3.0	64	1.5	1.5	12	>256	3.0	3.0
pH 7.5	3.0	6.0	1.0	1.0	8.0	32	1.5	1.5	32	>256	1.5	2.0	>256	>256	3.0	3.0
pH 7.0	>256	>256	1.0	1.0	>256	>256	1.5	1.5	>256	>256	1.5	2.0	>256	>256	3.0	3.0
pH 6.5	>256	>256	2.0	2.0	>256	>256	4.0	3.0	>256	>256	2.0	3.0	>256	>256	3.0	3.0
pH 6.0	>256	>256	2.0	3.0	>256	>256	6.0	5.0	>256	>256	2.0	3.0	>256	>256	3.0	3.0
Iso-Sensitest	0.38	6.0	0.5	0.75	0.75	8.0	1.5	1.5	1.0	12	2.0	2.0	2.0	>64	3.0	3.0

* As measured by "E" test.

Table 2. *Effect of cultural conditions on β-lactamase activity in P. cepacia (PC7)*

Cultural conditions	Enzyme activity (μmol/mg protein)	Isoelectric point(s)*			I_{50} value (μg/mL)	
					Clavulanate	Tazobactam
HMBB (in air or CO_2)						
pH 8.0	0.034	(6.5)		8.2	2.6	1.3
pH 7.5	0.029	(6.5)	(7.9)	8.2	2.5	1.1
pH 7.0	0.022	(6.5)	7.9	8.2	2.3	1.7
pH 6.5	0.021	(6.5)	7.9	8.2	2.3	1.9
pH 6.0	0.032	(6.5)	7.9	8.2	2.3	2.3
Iso-Sensitest agar						
Air	0.002	7.7			1.8	1.8
5% CO_2 in air	0.020	(6.5) 7.7		8.2	1.0	0.5
Air + imipenem (2 mg/L)	0.364	6.5 7.7 9.0	7.9 9.2	8.2	2.3	1.8

* Numbers in parentheses represent trace.

Table 3. *Hydrolysis profiles* of sonicates from P. cepacia (PC7) grown in air, 5% CO_2 in air and agar containing 2.0 mg/L imipenem*

β-Lactam (μg/disc)	Control zone diameter (mm) (no sonicate)	Residual zone diameters (mm) of discs pretreated with sonicates derived from O_2, CO_2, or air plus imipenem		
		O_2	CO_2	Air plus imipenem (2 mg/L)
Penicillin (1)	40	37	10	8
Methicillin (5)	33	32	11	9
Ampicillin (25)	26	28	16	<6
Ampicillin plus clavulanate (20:10)	28	28	28	28
Ticarcillin (75)	36	34	34	<6
Carbenicillin (75)	35	36	33	<6
Mezlocillin (75)	34	32	32	<6
Azlocillin (75)	28	29	28	20
Piperacillin (75)	38	36	34	33
Cephaloridine (25)	20	20	<6	<6
Cephalothin (30)	22	22	<6	<6
Cephradine (30)	16	16	9	<6
Cephalexin (30)	19	19	16	<6
Cefuroxime (30)	30	32	<6	<6
Ceftizoxime (30)	46	46	46	<6
Cefotaxime (30)	40	38	36	16
Ceftazidime (30)	36	36	34	36
Aztreonam (30)	38	38	40	24

* By disc (6 mm) inactivation method [Hart and Percival (4)] with *S. aureus* (NCTC 6571) or *E. coli* (NCTC 10418) as indicator organisms.

it is unlikely that these conditions are present at all sites of infection in the body.

In the present study the activities of ceftazidime, piperacillin, piperacillin/tazobactam, and cefpirome against *P. cepacia* were greatly decreased when it was grown in 5% CO_2 in air rather than in air alone. Although the MIC of these antibiotics increased by 10- to 30-fold when grown on Iso-Sensitest agar in CO_2, the MIC of ceftazidime (6 mg/L), piperacillin (8 mg/L), and piperacillin/tazobactam (12 mg/L) were still below the cutoff for resistance (16 mg/L). However, bacterial growth in air produced a surface pH of 9.05 on agar, which was decreased to pH 8.2 when grown in 5% CO_2 in air. When buffered growth medium was used, bacterial growth did not result in a change in pH of the medium whether or not the atmosphere was enriched with CO_2. At pH 7.5 or 8.0, incubation in the presence of CO_2 increased MIC, and at pH 7.5, piperacillin (MIC 32 mg/L) and piperacillin/tazobactam (MIC > 256 mg/L) were rendered clinically inactive. At pH 7.0 or below, each of the four agents had MIC greater than 256 mg/L whether or not the atmosphere was enriched with CO_2. Such dramatic changes were not observed when *P. aeruginosa* was grown under these various conditions. This finding indicates first that the effect is specific to *P. cepacia* and second that the effect is not due to instability of the β-lactams at different pH and CO_2 concentrations. Mechanisms of resistance to β-lactams include enzymic degradation of the antibiotic by β-lactamases or exclusion of the antibiotic from its site of action by impermeability. In the latter case, resistance to β-lactams in *P. cepacia* has been shown to be associated with mutations of outer membrane protein (porins) genes (18). We were unable to detect any change in expression of outer membrane proteins, in particular in the 36 kD porin in our isolates under the different growth conditions.

In contrast, incubation of each of the *P. cepacia* isolates in a CO_2-enriched atmosphere resulted in an increased expression of β-lactamase activity. For two isolates this resulted only in a 50% increase in β-lactamase activity; however, both these isolates had higher basal rates of enzyme production. Incubation of our standard strain, PC7 in 5% CO_2 in air, resulted in a 10-fold increase in β-lactamase activity. This increase was accompanied by the bacterium becoming resistant to most β-lactam antibiotics. The β-lactamase was also inducible by imipenem but to a much greater extent. The β-lactamase induced by growth in a CO_2-enriched atmosphere was able to inactivate 1st- and 2nd-generation cephalosporins but not 3rd-generation cephalosporins such as ceftazidime or cefotaxime. On isoelectric focusing, *P. cepacia* PC7 was found to produce one β-lactamase band of pI 7.7 when grown in air, whereas in CO_2-enriched air three bands of pI 6.5, 7.7, and 8.2 were seen. Induction by subinhibitory concentrations of imipenem (2 mg/L) resulted in hyperproduction of β-lactamase and new β-lactamase bands. In particular a β-lactamase of pI 7.9 was produced by imipenem induction and growth at pH < 7.5. β-Lactamases with pIs in this range in *P. cepacia* have been reported but not fully characterized previously (14). Our β-lactamase(s) has the characteristics of a cephalosporinase, and all enzyme activity, regardless of inducing conditions, was inhibitable by clavulanate and tazobactam. This places the β-lactamase in group 2e (cephalosporinases inhibited by low concentrations of clavulanic acid) in the classification scheme as described by Bush (19). A β-lactamase capable of hydrolyzing cefuroxime but inhibitable by clavulanate has been described previously in *P. cepacia*, but this enzyme has a pI of 9.3 (20).

Hyperproduction of β-lactamase does not completely explain the development of resistance to β-lactams after growth of *P. cepacia* in CO_2-enriched air because the enzyme could not inactivate ceftazidime or piperacillin. Although we were able to detect no quantitative change in the expression of the porin, this

would not preclude minor structural changes that might lead to a decrease in pore size and thus impermeability (21). However, the 4th-generation cephalosporin cefpirome orientates with a narrow cross-sectional area and would be expected to be relatively unaffected by decreases in porin size. Incubation of *P. cepacia* in CO_2 or at low pH greatly decreased the efficacy of cefpirome. It is possible, however, that a combination of changes in porins and hyperproduction of β-lactamase could act synergistically to enhance resistance to β-lactams (22).

From the foregoing it is clear that *in vitro* tests of antimicrobial susceptibility alter under different growth conditions. In the present study growth of *P. cepacia* in CO_2-enriched air or a pH < 7.5 resulted in induction of β-lactamase activity and development of resistance to most β-lactams. The atmospheric concentration of CO_2 is 0.003%, whereas that in the alveoli is 5–6%, similar to the concentration used by us to induce β-lactam resistance and β-lactamase production. Little information exists on alveolar CO_2 concentrations in cystic fibrosis; however, it is known that exertional hypercapnia occurs in patients with advanced stage cystic fibrosis as a result of chronic airflow limitation (23). Thus, it is possible that patients with advanced cystic fibrosis and *P. cepacia* infection might have even higher alveolar CO_2 concentrations. Expectorated sputum from patients with acute exacerbations of chronic bronchitis were found to have an average pH of 7.8 (24). However, endobronchial pH in patients with gram-negative pneumonia was found to average 6.61, and endobronchial pH in patients with chronic lung disease and in normal subjects were 6.62 and 6.58, respectively (25). Unfortunately, no information on sputum pH is available for patients with cystic fibrosis. However, it does seem likely the conditions of pH and CO_2 concentration at the site of *P. cepacia* infection in patients with cystic fibrosis are likely to result for our 15 isolates in hyperproduction of β-lactamases and thus clinical resistance to β-lactam antibiotics. It is noteworthy that despite conventional *in vitro* sensitivity to ceftazidime (MIC < 4 mg/L), of 18 episodes of *P. cepacia* infection in patients with cystic fibrosis treated with intravenous ceftazidime, only six resulted in clinical improvement and in only one case was the number of bacteria decreased (3).

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