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β-Lactamases and Detection of β-Lactam Resistance in *Enterobacter* spp.

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***Enterobacter* spp. are becoming increasingly frequent nosocomial pathogens, and β-lactam-resistant strains are on the increase, especially among isolates recovered from intensive care units. Therefore, a study was designed to characterize the β-lactamases produced by 80 isolates of *E. cloacae*, *E. aerogenes*, *E. taylorae*, *E. gergoviae*, *E. sakazakii*, *E. asburiae*, and *E. agglomerans* by induction studies, spectrophotometric hydrolysis assays, and isoelectric focusing. The ability of broth microdilution and disk diffusion susceptibility tests to detect resistance to 16 β-lactam antibiotics among these species was also assessed. All species except *E. agglomerans*, *E. gergoviae*, and some isolates of *E. sakazakii* were found to produce a Bush group 1 cephalosporinase that was expressed inducibly or constitutively at high levels. In addition, some strains also produced a Bush group 2 β-lactamase. In comparisons of broth microdilution and disk diffusion tests, disk diffusion tests failed to detect resistance in 1 of 25 isolates resistant to aztreonam and 2 of 30 isolates resistant to ceftazidime. These results indicate that species of *Enterobacter* can possess a variety of β-lactamases that are responsible for β-lactam resistance in this genus and that the disk diffusion test may occasionally miss resistance in some strains.**

The most recent edition of the *Manual of Clinical Microbiology* lists 14 species or biogroups of *Enterobacter* (11). Although not all of these have been implicated as causes of disease in humans, *Enterobacter aerogenes* and *Enterobacter cloacae* are commonly encountered nosocomial pathogens (2, 4, 7, 19, 35, 41). *Enterobacter agglomerans* (now *Pantoea agglomerans* [26]), *Enterobacter sakazakii*, *Enterobacter taylorae* (synonymous with *Enterobacter cancerogenus* [36]), *Enterobacter gergoviae*, and *Enterobacter asburiae* are only rare human pathogens, and infections with these species are usually associated with the presence of special risk factors (4, 7, 13, 19, 35). Numerous studies have shown that patients at increased risk of acquiring an *Enterobacter* infection include those with a prolonged hospital stay, especially if part of the stay is spent in an intensive care unit (1, 4, 15–17, 23, 28, 35). Other risk factors include the presence of a serious underlying illness, immunosuppression from any cause, extremes of age, the presence of a foreign device, and prior use of antimicrobial agents in the patient involved (35).

Considering the risk factors associated with acquisition of an *Enterobacter* infection, it is not surprising that the occurrence of such infections has significantly increased since the mid-1980s (22, 35). Although species of *Enterobacter* are responsible for 5 to 7% of all nosocomial bacteremias in the United States, this genus is the third most common pathogen recovered from the respiratory tracts of patients in intensive care units and the fifth most common pathogen recovered from the urinary tracts of such patients (22). Recovery of antibiotic-resistant strains of *Enterobacter* is also on the increase (5, 10, 24, 35, 38, 42). In general, the larger the hospital, the greater the prevalence of resistance among isolates of *Enterobacter* to β-lactam antibiotics, trimethoprim-sulfamethoxazole, and quinolones (5, 24, 35, 42). In a recent survey involving 144 hospitals across the United States, resistance to ceftazidime among *Enterobacter* spp. was found to be associated with isolates recovered from patients in intensive care units, isolates recovered from patients hospitalized between 1990 and 1991, and isolates recovered from blood or urinary tract infections (5).

Since the prevalence of *Enterobacter* spp. as a nosocomial pathogen is on the increase and the prevalence of β-lactam-resistant strains of this genus is on the rise, a study was designed to characterize the β-lactamases encountered in clinical isolates of various species of *Enterobacter*. The resistance phenotypes associated with each of the major types of β-lactamases found were also identified. The ability of the broth microdilution and disk diffusion tests used routinely in clinical microbiology laboratories to detect β-lactam resistance in isolates of *Enterobacter* was also assessed.

MATERIALS AND METHODS

Bacterial strains. Eighty isolates of the genus *Enterobacter* were selected for the study and included the following species: 30 strains of *E. cloacae*, 26 strains of *E. aerogenes*, 8 strains of *E. sakazakii*, 2 strains of *E. asburiae*, 6 strains of *E. taylorae*, 5 strains of *E. agglomerans*, and 3 strains of *E. gergoviae*. These strains were not random clinical isolates but were selected from collections on hand at BioMérieux Vitek Inc., St. Louis, Mo., and at the Center for Research in Anti-Infectives and Biotechnology, Omaha, Nebr. Priority for inclusion in this study was given to strains representing less frequently encountered species or those with unusual resistances. The strain of *E. cloacae* producing the NMC-A β-lactamase was kindly provided by P. Nordmann. For some species, resistant strains were not found among clinical isolates. Therefore, spontaneous mutants were selected in the laboratory by incubating the clinical isolates overnight in super-inhibitory concentrations of an expanded-spectrum cephalosporin such as ceftazidime.

Susceptibility testing. Antibiotic susceptibilities were determined by standard disk diffusion (31) and broth microdilution (30) procedures. Disks were obtained from Becton Dickinson Microbiology Systems (Cockeysville, Md.). Susceptibilities to the following β-lactam antibiotics were determined: ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, aztreonam, cephalothin, cefazolin, cefoxitin,

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TABLE 1. Phenotypes and susceptibility profiles for *E. aerogenes* obtained in broth microdilution tests

Phenotype[b]	No. of strains[c]	Provisional β-lactamase[d]	pI range	Susceptibility profiles[a]								
				sam	tic	tim	pip	tzp	caz	ctx	atm	imi
WT	4	Bush group 1	8.4–8.8	S/I	S	S	S	S	S	S	S	S
WT + OSBL	2	Bush group 1	8.4	R	R	R	R	S/R	S	S	S	S
		+										
		Bush group 2b (TEM-1)	5.4									
WT + ESBL	2	Bush group 1	8.4–>9	S	R	S/I	I/R	S	I/R	S/R	S/I	S
		+										
		Bush group 2be (TEM-3)	6.4									
		Bush group 2be (SHV-4)	7.8									
WT + OSBL + ESBL	1	Bush group 1	8.1	R	R	R	R	I	R	S	R	S
		+										
		Bush group 2b (TEM-1)	5.4									
		Bush group 2be (SHV-4)	7.8									
HYP	3	Bush group 1	>9	R	I	R	I	I	I/R	I	S/I	S
Mutant	14 (1)	Bush group 1	8.3–8.9	R	R	R	R	R	R	R	R	S

[a] S, susceptible; I, intermediate; R, resistant; drug abbreviations and NCCLS breakpoints for susceptibility/intermediate/resistance (in micrograms per milliliter), respectively, are as follows: ampicillin-sulbactam (sam), ≤8/16/≥32; ticarcillin (tic), ≤16/32 to 64/≥128; ticarcillin-clavulanic acid (tim), ≤16/32 to 64/≥128; piperacillin (pip), ≤16/32 to 64/≥128; piperacillin-tazobactam (tzp), ≤16/32 to 64/≥128; aztreonam (atm), ≤8/16/32; cefotaxime (ctx), ≤8/16 to 32/≥64; ceftazidime (caz), ≤8/16/≥32; and imipenem (imi), ≤4/8/≥16.

[b] Phenotypes are explained in Materials and Methods. WT, wild type; HYP, hyperinducible.

[c] Number of mutants selected in the laboratory is indicated in parentheses.

[d] Based on the classification of Bush et al. (6). β-Lactamase most similar to the one observed in the strain is indicated in parentheses.

cefotaxime, cetriaxone, ceftazidime, cefepime, and imipenem. For quality control purposes, the following quality control strains were run simultaneously with the test organisms: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 35218, and *Staphylococcus aureus* ATCC 29213. Throughout this study, results were interpreted by using the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) for disk diffusion (31) and broth dilution (30) tests. Custom-made broth microdilution panels were obtained from Pasco Laboratories, a division of Difco Laboratories (Detroit, Mich.).

β-Lactamase studies. Overnight cultures in Mueller-Hinton broth were diluted with 95 ml of fresh broth and were incubated with shaking for 90 min at 37°C. Cefoxitin, at a concentration of one-fourth of the MIC, was added for induction, while sterile medium was used in the noninduced cultures. Both cultures were incubated for a further 2 h. The induction process was stopped by adding 1 mM 8-hydroxyquinoline solution to each culture. Cells were harvested by centrifugation at 4°C, washed with 1 M potassium phosphate buffer (pH 7.0), suspended, and sonicated. After sonication, crude extracts were obtained by centrifugation at 5,858 × *g* for 1 h. The β-lactamases in the sonic extracts were assessed for pIs, substrate and inhibitor profiles in polyacrylamide gels, and rates of hydrolysis of cephalothin by UV spectrophotometric assay (3, 27, 32, 34). The three-dimensional test was performed to detect the presence of β-lactamases in strains for which no enzymes were detected on isoelectric focusing (IEF) gels. This is a modification of disk diffusion tests that yields β-lactamase substrate profile information (39). IEF of sonicates prepared from *E. gergoviae*, some isolates of *E. agglomerans*, and *E. sakazakii* initially revealed no β-lactamase bands. After confirming the presence of a β-lactamase in these sonicates by the three-dimensional test, the enzyme preparations were concentrated by negative pressure dialysis with a Micro-prodicon dialysis unit from Bio-Molecular Dynamics (Beaverton, Oreg.). The enzymes were then visualized by IEF.

Grouping of β-lactamases and phenotypes. For the purpose of this study, β-lactamases were placed into the major Bush groups (6) on the basis of the characteristics identified in this study or previously by other investigators. An enzyme was considered to belong to (i) Bush group 1 if on IEF it was susceptible to inhibition by cloxacillin but not clavulanic acid (34) or (ii) Bush group 2 if on IEF it was susceptible to inhibition by clavulanic acid but not cloxacillin (34). No enzymes similar to Bush group 3 or 4 were encountered in the collection studied. Enzymes belonging to Bush group 2 were provisionally designated (i) original-spectrum β-lactamases (OSBLs) if on IEF they possessed pIs identical to those of known Bush group 2b β-lactamases (e.g., TEM-1 and SHV-1) run on the same gel and did not hydrolyze cefotaxime in gel overlays (3) and (ii) extended-spectrum β-lactamases (ESBLs) if on IEF they possessed pIs identical to those of known Bush group 2be β-lactamases (e.g., TEM-3) run on the same gel and hydrolyzed cefotaxime in gel overlays. The phenotypes included wild type (an inducible Bush group 1 β-lactamase was present), basal (a Bush group 1 β-lactamase that was not inducible was expressed at a low level), derepressed mutant (a Bush group 1 enzyme produced at a high level either constitutively or semiconstitutively), and hyperinducible (a Bush group 1 β-lactamase produced inducibly but induced levels greatly exceeded those observed with the wild type).

RESULTS

Characteristics of *Enterobacter* β-lactamases. Most of the species of the genus *Enterobacter* were found to possess a Bush group 1 β-lactamase that had an alkaline pI ranging from 7.4 to greater than 9 and that was inhibited by cloxacillin but not clavulanic acid (Tables 1, 2, and 3). These enzymes were usually inducible in all species except *E. gergoviae*, *E. agglomerans*, and two isolates of *E. sakazakii* (Table 3). In these organisms, the enzyme was expressed constitutively at a low level, and this was designated the basal phenotype. Some strains of *E. aerogenes*, *E. cloacae*, *E. gergoviae*, and *E. agglomerans* also produced various Bush group 2 β-lactamases as well, and those β-lactamases were inhibited by clavulanic acid but not cloxacillin (Tables 1, 2, and 3).

Two isolates of *E. agglomerans* produced an enzyme that had a pI of 7.7, that did not hydrolyze the expanded-spectrum cephalosporins, that was inhibited by clavulanic acid but not cloxacillin, and that was thus similar to certain Bush group 2 OSBLs (Table 3). These organisms did not produce a detectable group 1 enzyme.

Susceptibility profiles. The antimicrobial susceptibility profiles for the various phenotypes observed with each species are presented in Tables 1, 2, and 3. Only results obtained with those drugs that helped to differentiate between the phenotypes are presented. For *E. aerogenes* and *E. cloacae*, the major difference between the wild-type and the wild-type plus OSBL phenotypes was resistance to penicillins and inhibitor-drug combinations in strains of the latter phenotype. In these spe-

TABLE 2. Phenotypes and susceptibility profiles for *E. cloacae* obtained in broth microdilution tests

Phenotype[b]	No. of strains[c]	Provisional β-lactamase[d]	pI range	Susceptibility profiles[a]								
				sam	tic	tim	pip	tzp	caz	ctx	atm	imi
WT	6	Bush group 1	8.0–>9	I/R	S/I	S	S	S	S	S	S	S
WT + OSBL	4	Bush group 1	7.9–>9	R	R	R	R	R	S	S	S	S
		+										
		Bush group 2b (TEM-1)	5.4									
WT + ESBL	3	Bush group 1	7.9–>9	S/I/R	R	S/I	I/R	S	S/I	S/I/R	S/I	S
		+										
		Bush group 2be (TEM-3)	6.4									
		Bush group 2be (SHV-3)	6.8									
WT + OSBL + ESBL	1	Bush group 1	>9	R	R	R	R	S	S	S	S	S
		+										
		Bush group 2b (TEM-1)	5.4									
		Bush group 2be (SHV-2)	7.6									
WT + CPEN	1	Bush group 1	>9	R	S	S	S	S	S	S	S	R
		+										
		Bush group 2 (NMC-A)	6.7									
Mutant	12 (4)	Bush group 1	7.8–>9	R	R	R	R	R	R	R	R	S
Mutant + OSBL	3	Bush group 1	>9	R	R	R	R	R	R	R	R	S
		+										
		Bush group 2b (TEM-1)	5.4									

[a] S, susceptible; I, intermediate; R, resistant. Drug abbreviations: amp, ampicillin-sulbactam; tic, ticarcillin; tim, ticarcillin-clavulanic acid; pip, piperacillin; tzp, piperacillin-tazobactam; atm, aztreonam; ctx, cefotaxime; caz, ceftazidime; and imi, imipenem. See footnote *a* of Table 1 for NCCLS breakpoints.
[b] Phenotypes are explained in Materials and Methods. WT, wild type; CPEN, carbapenemase.
[c] Number of mutants selected in the laboratory is indicated in parentheses.
[d] Based on the classification of Bush et al. (6). The β-lactamase most similar to the one observed in the strain is indicated in parentheses.

cies, the wild-type plus ESBL phenotype characteristically showed decreased susceptibility or resistance to one or more of the expanded-spectrum cephalosporins and/or aztreonam but remained susceptible to piperacillin-tazobactam (Tables 1 and 2). Derepressed mutants and hyperinducible mutants of these species were usually resistant to all drugs tested except cefepime and imipenem. There were too few strains with the wild-type plus OSBL plus ESBL phenotype to ascertain whether any susceptibility profile was peculiar to this phenotype (Tables 1 and 2).

For the other species of *Enterobacter* examined, derepressed mutants could be clearly differentiated from strains with the wild-type phenotype by the greater resistance of the former to a variety of β-lactam antibiotics (Table 3). Those strains with a basal phenotype had a susceptibility profile similar to that of strains with the wild-type phenotype with the exception of *E. sakazakii*. For this species, strains with a basal phenotype were susceptible to cefoxitin, while those with the wild-type phenotype were resistant. The presence of an OSBL in these species increased resistance to some penicillins and inhibitor-drug combinations (Table 3).

Susceptibility testing. In comparisons of broth microdilution and disk diffusion tests by using the broth microdilution test as the standard, the disk diffusion test failed to detect resistance in 1 of 25 isolates resistant to aztreonam and 2 of 30 isolates resistant to ceftazidime. Furthermore, 3 of 33 isolates susceptible to piperacillin-tazobactam, 1 of 21 isolates susceptible to ticarcillin-clavulanate, and 1 of 34 isolates susceptible to cefotaxime by the broth microdilution test appeared resistant by disk diffusion.

DISCUSSION

Antimicrobial susceptibility within the genus *Enterobacter* varies widely due to the diverse species within the genus. This was reported earlier by Muytjens and van der Ros-van de Repe (29) in one of the most complete analyses of differences in antimicrobial susceptibility between various species of *Enterobacter*. Those investigators examined the activities of 29 antimicrobial agents against eight species of *Enterobacter*. Their results showed that some strains of *E. sakazakii* and *E. agglomerans* are sensitive to ampicillin, cephalothin, and cefoxitin, three β-lactam drugs to which *E. cloacae* and *E. aerogenes* are resistant. Our study supported the findings from Muytjens and van der Ros-van de Repe (29). The different susceptibility patterns among *E. cloacae* and *E. aerogenes* in our study were similar to those reported by Muytjens and van der Ros-van de Repe (29), although *E. cloacae* tended to be more resistant than *E. aerogenes* to the different β-lactam drugs tested. The wild-type strains of *E. cloacae*, *E. aerogenes*, *E. taylorae*, and *E. asburiae* were uniformly resistant to ampicillin, cefoxitin, and the narrow-spectrum cephalosporins, while most wild-type strains of *E. sakazakii*, *E. agglomerans*, and *E. gergoviae* were sensitive to these drugs. This was in accordance with the report from Muytjens and van der Ros-van de Repe (29) as well as those from other investigators (12, 14, 20, 25, 40). The type of β-lactamases present in wild-type strains of *E. cloacae*, *E. aerogenes*, *E. taylorae*, and *E. asburiae* could explain the differences in susceptibility between these species and *E. gergoviae*, *E. sakazakii*, and *E. agglomerans*. The former species produced an inducible Bush group 1 β-lactamase, whereas enzyme production in *E. gergoviae*, *E. agglomerans*, and some strains of *E.*

TABLE 3. Phenotypes and susceptibility profiles obtained in broth microdilution tests with *Enterobacter* spp. other than *E. cloacae* and *E. aerogenes*

Phenotype[b]	No. of strains[c]	Provisional β-lactamase[d]	pI range	Susceptibility profiles[a]									
				sam	tic	tim	pip	tzp	caz	ctx	fox	atm	imi
E. gergoviae													
Basal	2	Bush group 1	>9	S/I	S/I	S/I	S	S	S	S	S/R	S	S
Basal + OSBL	1	Bush group 1 + Bush group 2b (TEM-1)	>9 5.4	R	R	I	S	S	S	S	R	S	S
E. tayloriae													
WT	4	Bush group 1	>9	S/R	S/I	S/I	S	S	S	S	R	S	S
Mutant	2 (2)	Bush group 1	>9	R	R	R	I	I/R	R	I/R	R	I/R	S
E. sakazakii													
WT	3	Bush group 1	8.8–>9	S	S	S	S	S	S	S	R	S	S
Basal	2	Bush group 1	7.4–8.0	S	S	S	S	S	S	S	S	S	S
Mutant	3 (2)	Bush group 1	8.8–>9	R	I/R	I/R	I/R	I/R	R	I/R	R	I/R	S
E. agglomerans													
Basal	2	Bush group 1	7.7–>9	S/I	S/I	S	S	S	S	S	S	S	S
Basal + OSBL	1	Bush group 1 + Bush group 2b (TEM-1)	>9 5.4	R	R	R	R	R	S	S	R	S	S
OSBL	2	Bush group 2	7.7	S	S/R	S	S	S	S	S	S/R	S	S
E. asburiae													
WT	1	Bush group 1	>9	R	S	S	S	S	S	S	R	S	S
Mutant	1	Bush group 1	>9	R	R	R	R	R	R	R	R	R	S

[a] S, susceptible; I, intermediate; R, resistant. Drug abbreviations: sam, ampicillin-sulbactam; tic, ticarcillin; tim, ticarcillin-clavulanic acid; pip, piperacillin; tzp, piperacillin-tazobactam; atm, aztreonam; ctx, cefotaxime; caz, ceftazidime; fox, cefoxitin (breakpoints: susceptibility, ≤8 μg/ml; intermediate, 16 μg/ml; resistance, ≥32 μg/ml); and imi, imipenem.
[b] Phenotypes are explained in Materials and Methods. WT, wild type.
[c] Number of mutants selected in the laboratory is indicated in parentheses.
[d] Based on the classification of Bush et al. (6). The β-lactamase most similar to the one observed in the strain is indicated in parentheses.

sakazakii was at a lower level and was not inducible with cefoxitin, cephalothin, or ampicillin. Derepressed mutants of *E. cloacae*, *E. aerogenes*, *E. taylorae*, *E. sakazakii*, and *E. asburiae* produced the Bush group 1 β-lactamase constitutively at high levels, which was reflected in resistance to most β-lactam antibiotics except cefepime and imipenem.

E. agglomerans has recently been reclassified as *Pantoea* spp. (26). We examined five isolates of these species and found three different phenotypes. This included two isolates producing enzymes with pIs of 7.7 resembling Bush group 2 β-lactamases on IEF. These observations support the contention that *E. agglomerans* represents a taxonomically heterogeneous group of diverse organisms and is genetically distant from other species of *Enterobacter*.

Enterobacter spp. producing ESBLs have been included in this study and have been recently isolated in France (8, 9, 18), the United States (33), and the United Kingdom (21). It is important to detect these strains in a clinical laboratory and to differentiate them from derepressed mutants. Plasmids encoding ESBLs may also encode resistance to other classes of antibiotics such as the aminoglycosides and trimethoprim-sulfamethoxazole, limiting the treatment options of physicians facing patients with infections caused by organisms producing these enzymes (37). Therefore, factors leading to the selection and spread of strains producing ESBLs need to be identified and, where possible, eliminated (37). In this study the derepressed mutants of *E. cloacae* and *E. aerogenes* were uniformly resistant to piperacillin-tazobactam, expanded-spectrum cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), and aztreonam, while wild-type strains of *E. cloacae* and *E. aerogenes* producing ESBLs remained sensitive to piperacillin-tazobactam and showed decreased susceptibility to either cefotaxime, ceftriaxone, ceftazidime, or aztreonam but not necessarily to all of these antibiotics. The pattern of resistance to the expanded-spectrum cephalosporins and aztreonam depended on the type of ESBL produced. All strains producing ESBLs in this study showed resistance to a combination of cefotaxime, ceftriaxone, ceftazidime, and aztreonam. Thus, for a clinical laboratory to effectively detect species of *Enterobacter* producing ESBLs, a combination of piperacillin-tazobactam, cefotaxime, or ceftriaxone with ceftazidime and aztreonam should be included in the test panel for routine susceptibility testing.

Enterobacter spp. are important nosocomial pathogens. Because of the popularity of the expanded-spectrum cephaloporins, they will probably continue to increase in prevalence. Thus, the challenge to clinicians and microbiologists to recognize susceptibility patterns indicative of the presence of specific β-lactamases will become even more important as the members of this genus acquire additional antimicrobial resistance in the future.

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