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Resistance to Cefepime and Cefpirome Due to a 4-Amino-Acid Deletion in the Chromosome-Encoded AmpC β-Lactamase of a *Serratia marcescens* Clinical Isolate

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A multiresistant *Serratia marcescens* strain, HD, isolated from a patient with a urinary tract infection, was resistant to amino-, carboxy-, and ureidopenicillins, ceftazidime, and cefepime and was susceptible to cefotaxime and ceftriaxone, according to the guidelines of the NCCLS. No synergy was found between expanded-spectrum cephalosporins and clavulanic acid, according to the double-disk synergy test. The bla_{AmpC} gene of the strain was amplified by PCR and cloned into *Escherichia coli* DH10B, giving rise to high-level resistance to ceftazidime, cefepime, and cefpirome. Sequencing analysis revealed that the bla_{AmpC} gene from *S. marcescens* HD had a 12-nucleotide deletion compared to the bla_{AmpC} gene from reference strain *S. marcescens* S3, leading to a 4-amino-acid deletion located in the H-10 helix of the β-lactamase. Kinetic analysis showed that this enzyme significantly hydrolyzed ceftazidime, cefepime, and cefpirome. This work underlined that resistance to the latest expanded-spectrum cephalosporins may be mediated by structurally modified AmpC-type β-lactamases.

Serratia marcescens is a saprophytic, water-borne gram-negative rod (7) that possesses an inducible, chromosomally encoded AmpC-type β-lactamase (11) and is often involved in nosocomial infections (5, 8, 16, 19). Infections caused by cephalosporinase-producing *Enterobacteriaceae* may be difficult to treat due to their ability to confer resistance to a variety of β-lactams, including the expanded-spectrum cephalosporins cefotaxime and ceftazidime. This resistance may arise from at least three different mechanisms in *S. marcescens*: high-level production of the chromosomal AmpC-type cephalosporinase (9), acquisition of an Ambler class A extended-spectrum β-lactamase (21), and acquisition of metallo-β-lactamases (21). Cefepime and cefpirome, which contain quaternary nitrogen atoms at three different positions, remain active against overexpressed AmpC-producing enterobacteria. This activity results from both the stability of these molecules to attack by cephalosporinase (2, 6) and their rapid penetration through the outer membrane (15, 18).

We analyzed an *S. marcescens* clinical isolate harboring a chromosomally encoded β-lactamase with β-lactam hydrolysis extended to a cefepime that possessed a 4-amino-acid deletion compared to a wild-type cephalosporinase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Clinical isolate *S. marcescens* HD was identified by using the API 20E system (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* DH10B was used for the experiments (3). *S. marcescens* strain S3 was previously characterized as a wild-type AmpC producer (gift from H. Nikaido) (17).

Cloning of β-lactamase genes. Whole-cell DNAs of *S. marcescens* isolates HD and S3 were extracted as described previously (3). On the basis of the *ampC* gene sequence of *S. marcescens* S3 (17), primers PRESM1 (5′-ATACCCTGCAACC TAAGAGC-3′) and PRESM2 (5′-ATCGCTGGTAGGGGCGCCTC-3′) were designed to amplify a 1,195-bp fragment corresponding to *ampC* genes without their own promoter sequence. The amplification products were ligated into pBK-CMV phagemid (Stratagene, Amsterdam, The Netherlands) that had previously been digested with the restriction enzyme *Sca*I (Amersham Pharmacia Biotech, Orsay, France). Whole-cell DNA of *S. marcescens* HD was also digested by *Hin*dIII, and DNA fragments were ligated into *Hin*dIII-restricted phagemid vector pBK-CMV. Recombinant phagemids were transformed into *E. coli* strain DH10B by electroporation with a Gene Pulser II apparatus (Bio-Rad, Ivry-sur-Seine, France). Transformants were selected on Trypticase soy agar containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml). Recombinant plasmids were purified with a plasmid Midi kit (QIAGEN, Courtaboeuf, France). The cloned β-lactamase genes were sequenced on both strands by using an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences were analyzed with software available over the Internet from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The ClustalW program (www.infobiogen.fr) was used for the alignment of multiple protein sequences.

IEF analysis. The β-lactamase extracts from cultures of clinical isolates and purified enzymes were subjected to analytical isoelectric focusing (IEF) analysis as previously described (3) by using an ampholine polyacrylamide gel with a pH range of 3.5 to 9.5 (ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with a 1 mM nitrocefin solution (Calbiochem, Merck Eurolab SAS, Fontenay-sous-bois, France).

Antimicrobial agents and MIC determination. The antibiotic agents and their sources have been described elsewhere (3). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Paris, France) with an inoculum of 10^4 CFU per spot and were interpreted according to the guidelines of the NCCLS (14).

β-Lactamase purification. Recombinant *E. coli* DH10B strains were grown overnight at 37°C in 4 liters of Trypticase soy broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml), resuspended in 40 ml of 100 mM phosphate buffer (pH 7), disrupted by sonication, and centrifuged at 20,000 × *g* for 1 h at 4°C as previously described (3). β-Lactamase extracts were filtered through a 0.45-μm-pore-size filter (Millipore, Saint-Quentin-en-Yvelines, France), dialyzed overnight at 4°C against 20 mM Tris (pH 7.5), and loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The flowthrough fractions containing the β-lactamase were recovered and dialyzed against 50 mM

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FIG. 1. Alignment of the amino acid sequences of the AmpC β-lactamases of *S. marcescens* HD, *S. marcescens* clinical isolate S3, *E. cloacae* P99 (10), and *E. cloacae* CHE (2). Asterisks indicate amino acid residues identical to those of the cephalosporinase AmpC HD of *S. marcescens* HD. The N-terminal amino acid of the mature enzymes is designated position 1. The amino acid sequence from positions −22 to 1 is assumed to be the signal peptide. Dashes show a deletion or the absence of an amino acid residue when the sequences were aligned after optimal matching of the entire protein. The serine β-lactamase motif S-L-S-K, the conserved triad K-T-G, and the Ambler class C typical motif Y-X-N are shaded.

phosphate buffer (pH 6) before being loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzymes were eluted by a linear NaCl gradient (0 to 1 M) in the same buffer. The eluted fractions with the highest β-lactamase activity (nitrocefin test) were pooled and dialyzed against 100 mM phosphate buffer (pH 7). To assess the purity of the extracts, purified enzymes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (3).

Kinetic measurements. Purified β-lactamases AmpC S3 and AmpC HD were used for kinetic measurements (K_m and k_{cat}), which were made at 30°C in 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with a Pharmacia ULTROSPEC 2000 spectrophotometer and were analyzed by using the SWIFT II software (Amersham Pharmacia Biotech). K_m and k_{cat} values were determined by analyzing β-lactam hydrolysis under initial rate conditions by using the Eadie-Hofstee linearization of the Michaelis-Menten equation as previously described (4). When the K_m value was very low, the K_i value was determined from the initial rates at saturating substrate concentrations ($[S] \gg K_m$). Values were the means of three independent measures (the standard deviations of the values were within 15%).

Nucleotide sequence accession number. The nucleotide sequence of the $bla_{\text{AmpC HD}}$ gene from *S. marcescens* isolate HD has been submitted to the GenBank nucleotide database under the accession number AY336102.

RESULTS

Isolation of the clinical isolate. *S. marcescens* clinical strain HD was isolated from a urine specimen of a patient 7 days after admission into a surgical ward of the Laennec Hospital (Nantes, France) in September 2001. This isolate was selected for further study on the basis of its uncommon pattern of susceptibility to β-lactam antibiotics, including resistance to cefepime. *S. marcescens* HD was susceptible to aminoglycosides and cotrimoxazole but was resistant to fluoroquinolones. The urinary tract infection was treated successfully with cotrimoxazole for 7 days. No cefepime treatment was given prior to the isolation of this strain.

Cloning and sequence analysis of β-lactamase genes from *S. marcescens* isolates HD and S3. DNA sequence analysis revealed a lack of 12 nucleotides in the sequence of the $bla_{\text{AmpC HD}}$ gene compared to that of the $bla_{\text{AmpC S3}}$ gene (wild-type bla_{AmpC} gene of *S. marcescens*), leading to a 4-amino-acid deletion, MNGT, at positions 233 to 236 of the protein (Fig. 1). Cloning experiments using PCR products with each of the *S. marcescens* isolates as templates yielded recombinant strains *E. coli* DH10B(pBK-HD) and *E. coli* DH10B(pBK-S3). The alignment of the sequences of these AmpC-type β-lactamases with those of the *Enterobacter cloacae* P99 and *E. cloacae* CHE isolates revealed that the position of amino acids MNGT matched that of amino acids SKVALA, which were deleted in the sequence of the extended-spectrum AmpC-type β-lactamase CHE (2) compared to that of the cephalosporinase of *E. cloacae* P99 (Fig. 1). The cloning experiment using whole-cell DNA of *S. marcescens* HD digested with *Hin*dIII gave another recombinant clone, *E. coli* DH10B(pBK-TEM-1); the DNA sequence analysis of this insert showed its identity with the $bla_{\text{TEM-1}}$ gene.

Susceptibility testing. The MICs of several β-lactams for *S. marcescens* isolates HD and S3 and their corresponding *E. coli* DH10B clones are reported in Table 1. Susceptibility data showed that *S. marcescens* HD was resistant to penicillins, narrow-spectrum cephalosporins, ceftazidime, and cefepime; had reduced susceptibility to cefoxitin; and was susceptible to cefotaxime, ceftriaxone, aztreonam, and imipenem. In comparison, *S. marcescens* S3 was resistant to amoxicillin and narrow-spectrum cephalosporins; had a decreased susceptibility to ticarcillin, piperacillin, and cefoxitin; and was susceptible to ceftazidime, cefotaxime, ceftriaxone, aztreonam, cefpirome, cefepime, and imipenem. *E. coli* DH10B(pBK-HD) remained susceptible to ticarcillin, aztreonam, and imipenem; had a reduced susceptibility to cefotaxime and ceftriaxone; and was resistant to the other β-lactams tested. MIC determinations for *E. coli* DH10B(pBK-S3) showed that the pattern of resistance of the recombinant strain mirrored that of the parental strain

TABLE 1. MICs of β-lactams for *S. marcescens* HD and S3 strains and for recombinant *E. coli* DH10B(pBK-HD) and *E. coli* DH10B(pBK-S3)

β-Lactam(s)[f]	MIC (μg/ml)					
	S. marcescens HD[a]	*S. marcescens* S3[b]	*E. coli* DH10B(pBK-HD)[c]	*E. coli* DH10B(pBK-S3)[d]	*E. coli* DH10B(pBK-TEM-1)[e]	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	>512	2
Amoxicillin-CLA	256	>512	256	256	512	2
Ticarcillin	>512	32	8	128	>512	1
Ticarcillin-CLA	256	32	8	128	>512	1
Piperacillin	512	16	256	256	64	1
Piperacillin-TZB	32	8	128	128	32	1
Cephalothin	>512	>512	>512	>512	8	4
Cefoxitin	8	16	128	128	2	2
Cefuroxime	128	512	512	128	0.5	0.5
Ceftriaxone	2	0.25	16	8	<0.06	<0.06
Cefotaxime	2	0.25	16	8	<0.06	<0.06
Ceftazidime	64	0.06	512	16	<0.06	<0.06
Aztreonam	0.5	0.06	0.25	0.06	0.06	0.06
Cefepime	32	0.06	512	2	0.06	0.06
Cefpirome	8	0.06	64	2	0.06	0.06
Imipenem	0.06	0.06	0.06	0.06	0.06	0.06

[a] *S. marcescens* isolate HD produced β-lactamases AmpC HD and TEM-1.
[b] *S. marcescens* isolate S3 reference strain produced β-lactamase AmpC S3.
[c] The recombinant *E. coli* DH10B(pBK-HD) strain produced β-lactamase AmpC HD.
[d] The recombinant *E. coli* DH10B(pBK-S3) strain produced β-lactamase AmpC S3.
[e] The recombinant *E. coli* DH10B(pBK-TEM-1) strain produced β-lactamase TEM-1.
[f] CLA, clavulanic acid at 2 μg/ml; TZB, tazobactam at 4 μg/ml.

S3, with a better expression of resistance to ticarcillin, piperacillin, and cefoxitin. Recombinant *E. coli* DH10B that expressed the $bla_{\text{TEM-1}}$ gene from *S. marcescens* HD had a typical clavulanate-inhibited phenotype (Table 1).

Biochemical analysis of β-lactamase AmpC HD from *S. marcescens* HD and β-lactamase AmpC S3 from *S. marcescens* S3. AmpC enzymes were purified to near homogeneity (>99%), as deduced from SDS-PAGE analysis (Fig. 2). The catalytic efficiency of the purified β-lactamase AmpC HD against the expanded-spectrum cephalosporins cefepime, cefpirome, and ceftazidime was at least 20-fold higher than that observed for the *S. marcescens* S3 enzyme (Table 2). This result was mostly due to a decrease in the K_m values for those substrates. However, the affinity of AmpC HD toward benzylpenicillin, amoxicillin, ticarcillin, and piperacillin was decreased compared to that of AmpC S3 (higher K_m values).

IEF analysis. IEF analysis of β-lactamase extracts of culture of *S. marcescens* HD gave two bands with pI values of 8.6 and 5.4 that comigrated with β-lactamases extracted from *E. coli* DH10B(pBK-HD), expressing the cephalosporinase variant, and *E. coli* DH10B(pBK-TEM-1), expressing TEM-1, respectively. Cultures of *S. marcescens* S3 and *E. coli* DH10B(pBK-S3) produced a single β-lactamase with a pI of 8.5 that corresponded to the wild-type cephalosporinase S3.

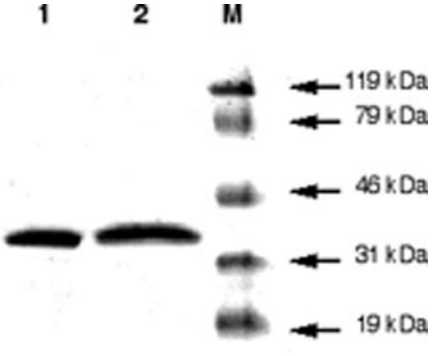

FIG. 2. SDS-PAGE analysis of purified β-lactamases from cultures of *E. coli* DH10B(pBK-HD) and *E. coli* DH10B(pBK-S3) expressing AmpC β-lactamases HD and S3, respectively. Lane 1, purified AmpC HD; lane 2, purified AmpC S3; lane M, molecular mass markers (size indicated to the right of the figure).

TABLE 2. Kinetic parameters of β-lactamases AmpC HD and AmpC S3

β-Lactam[a]	AmpC HD			AmpC S3		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
Benzylpenicillin	50	10	5,000	35	9	4,000
Amoxicillin	0.01	1	10	0.01	0.3	40
Ticarcillin	<0.001	2		0.002	0.05	50
Piperacillin	0.1	0.5	200	0.01	0.1	110
Cephalothin	95	2	48,000	1,200	125	9,600
Cefoxitin	0.01	3	2	0.002	1	2
Cefuroxime	4.5	5	1,000	2.5	7	350
Ceftriaxone	2	4	500	0.9	5	180
Cefotaxime	5	2	2,300	6	7	800
Ceftazidime	270	20	13,000	5	>1,000	<5
Cefepime	50	6	8,400	80	>1,000	<80
Cefpirome	270	100	2,800	120	>1,000	<120
Aztreonam	<0.001			<0.001		
Imipenem	<0.001			<0.001		

[a] For those compounds with K_m values less than 5 μM, K_i values were determined instead of K_m, with cephalothin as the substrate. The values are the means of results from at least three independent experiments.

DISCUSSION

This report describes an AmpC-type β-lactamase from *S. marcescens* that conferred resistance to cefepime. Rare AmpC-type β-lactamases of *S. marcescens* with an expanded spectrum of hydrolysis have been reported. Raimondi et al. (17) characterized an enzyme with an isoleucine-for-threonine substitution at position 64, conferring resistance to ceftazidime and cefpirome, although its activity against cefepime was not studied. Matsumura et al. (12) have characterized AmpC SRT-1, which has a lysine-for-glutamate substitution at position 213 compared to wild-type AmpC SST-1, which confers resistance to oxyimino cephalosporins. We report here for the first time an *S. marcescens* strain that is highly resistant to cefepime and cefpirome because of its production of an AmpC-type β-lactamase and that it is also a clinical isolate.

Sequence analysis of AmpC HD indicated that a deletion of 4 amino acids was involved in resistance to ceftazidime, cefepime, and cefpirome. The catalytic efficiencies (k_{cat}/K_m) of AmpC HD for these β-lactams were increased compared to those of β-lactamase AmpC S3, taken as the wild-type enzyme, mostly resulting from an increase of the affinity for these substrates. Moreover, DNA sequence analysis revealed that $bla_{AmpC\ HD}$ was the only cephalosporinase-encoding gene harbored by the *S. marcescens* HD isolate, suggesting that it was likely the natural, chromosomally encoded bla_{AmpC} gene of the *S. marcescens* strain that had undergone a 4-amino-acid deletion.

Four AmpC-type variants conferring resistance to cefepime have been described among AmpC β-lactamases of other enterobacterial species. The AmpC harbored by *E. cloacae* CHE had a 6-amino-acid deletion at positions 289 to 294 (2). A laboratory-obtained *E. coli* strain harboring AmpC-type β-lactamase of *E. cloacae* P99 had a proline-for-leucine substitution at position 293 (20). In vivo selection of an AmpC-type enzyme derived from the wild-type enzyme of *E. cloacae* strain MHN had a glutamic acid-for-valine substitution at position 298 (13). Finally, in vitro-obtained AmpC enzymes with several amino acid substitutions, including replacements at positions 292, 293, 294, 296, and 298 in the sequence of the plasmid-mediated CMY-2 β-lactamase (related to the chromosomally encoded AmpC of *Citrobacter freundii*), had resistance to cefepime (1). All these substitutions and deletions are located in the vicinity of the H-10 alpha helix, which is close to the C-terminal extremity of the protein and far from the active site located nearby the H-2 helix (10). These sequence changes may explain the extension of the substrate specificity, including that to cefepime, that was related to a decrease in the K_m.

However, no crystallographic analysis of the AmpC β-lactamase of *S. marcescens* is available. Once the amino acid sequences of AmpC β-lactamases of *S. marcescens* HD and *E. cloacae* CHE were superimposed, we assumed that the deletion observed in AmpC HD was also located in the H-10 alpha helix (2). β-Lactamases AmpC HD of *S. marcescens* HD and AmpC CHE of *E. cloacae* CHE shared the same overall activity toward cephalosporins, providing an increased resistance to cefepime, cefpirome, and ceftazidime, whereas cefotaxime was hydrolyzed weakly. However, β-lactamase AmpC HD of *S. marcescens* conferred on an *E. coli* transformant a level of cefepime resistance (MIC > 512 μg/ml) that was higher than that of the AmpC CHE (MIC = 8 μg/ml). We assumed that this discrepancy was due to a 10-fold-higher catalytic efficiency of the AmpC HD β-lactamase.

This work provides further evidence that the substrate specificity of natural, chromosomally encoded AmpC may evolve to confer resistance to cefepime and cefpirome, which may no longer be considered optimal β-lactams for treating infections due to enterobacterial isolates with overexpressed cephalosporinases. However, the nature of the genetic rearrangement found in the β-lactamase gene (a 12-nucleotide deletion) makes the selection of such *S. marcescens* strains at high frequency unlikely. Finally, it remains to be determined why deletion as a source of extension of the hydrolysis spectrum has been reported for class C β-lactamases and not for β-lactamases belonging to the other Ambler classes.

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