ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2003, p. 1297–1300 Vol. 47, No. 4
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.4.1297–1300.2003

Imipenem Resistance in a *Salmonella* Clinical Strain Due to Plasmid-Mediated Class A Carbapenemase KPC-2

Vivi Miriagou,[1] Leonidas S. Tzouvelekis,[2] Shannon Rossiter,[3] Eva Tzelepi,[1] Frederick J. Angulo,[3] and Jean M. Whichard[3]*

Laboratory of Bacteriology, Hellenic Pasteur Institute,[1] and Department of Microbiology, Medical School,[2] University of Athens, Athens, Greece, and Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[3]

Received 29 July 2002/Returned for modification 24 October 2002/Accepted 2 January 2003

A *Salmonella enterica* serotype Cubana isolate exhibiting resistance to most β-lactam antibiotics, including oxyimino-cephalosporins and imipenem, was isolated from a 4-year-old boy with gastroenteritis in Maryland. β-Lactam resistance was mediated by a conjugative plasmid that encoded KPC-2, a class A carbapenemase previously found in a *Klebsiella pneumoniae* isolate from the Maryland area as well. Sequence analysis of the flanking regions indicated a potential association of bla_{KPC-2} with mobile structures.

Resistance to expanded-spectrum oxyimino-cephalosporins among *Salmonella* strains is mostly due to acquisition of plasmids encoding various class A extended-spectrum β-lactamases (1, 8, 13, 17, 19–21). Production of plasmid-mediated class C β-lactamases by *Salmonella* isolates has also been described previously (4, 22, 23). The emergence of such strains may have serious implications because of the limitation of therapeutic choices for patients with invasive *Salmonella* infections and by facilitation of the spread of *bla* genes in the community. We describe here an imipenem-resistant *Salmonella enterica* serotype Cubana isolate that produces the recently described KPC-2 β-lactamase (E. S. Moland, J. Johnson, J. A. Black, T. J. Lockhart, A. Hossain, V. L. Herrera, N. D. Hanson, and K. S. Thomson, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2226, 2001).

MATERIALS AND METHODS

Salmonella serotype Cubana was isolated in December 1998 from a stool specimen of a 4-year-old boy with diarrhea in a hospital in Maryland. The patient was chronically ill with Wiskott-Aldrich syndrome. In the 6 months before isolation of serotype Cubana, he had been hospitalized three times and had received intravenous antibiotics. During each of those hospitalizations he had received intravenous β-lactams, including ceftriaxone and ceftazidime, but not carbapenems. There was no history of recent travel. The isolate (AM04707) was submitted to the Centers for Disease Control and Prevention as part of the National Antimicrobial Resistance Monitoring System for enteric bacteria (http://www.cdc.gov/NARMS/) and was subsequently forwarded to the Hellenic Pasteur Institute.

Escherichia coli K-12 strain 14R525 (Nal^r) was used as the recipient in conjugation experiments. *E. coli* DH5α (GIBCO-BRL, Carlsbad, Calif.) was used for transformation. Chloramphenicol-resistant plasmid pBCSK(+) (Stratagene, La Jolla, Calif.) was used for cloning and expression of *bla* genes.

The MICs of β-lactams were determined by an agar dilution technique (10). Susceptibilities to other antimicrobial agents were assessed by a disk diffusion method (11) and by a partial-range broth microdilution method (Sensititre; Trek Diagnostics; Westlake, Ohio), according to the instructions of the manufacturer.

Conjugation experiments were carried out in mixed broth cultures as described previously (5). Transconjugant clones were selected on Mueller-Hinton agar containing ampicillin (50 μg/ml) plus nalidixic acid (200 μg/ml). Plasmid DNA preparations were obtained by an alkaline lysis technique and resolved in 0.8% (wt/vol) agarose gels. Individual plasmids were excised as discrete bands from low-melting-point agarose (0.8%) and were subjected to partial digestion with various restriction enzymes including *Hin*dIII. Digests were ligated into the multicloning site of pBCSK(+). The resulting recombinant plasmids were used to transform *E. coli* DH5α competent cells. β-Lactam-resistant transformants were selected on Luria-Bertani agar containing chloramphenicol (20 μg/ml) and ampicillin (50 μg/ml). The nucleotide sequences of the cloned fragments were determined with an ABI Prism 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). Sequence similarity searches were performed with the BLAST program (available at the website of the National Center for Biotechnology Information).

β-Lactamases were extracted in one of two ways. One method involved ultrasonic treatment of bacterial cells, followed by suspension in phosphate buffer (100 mM; pH 7.0) and clarification by centrifugation (Fig. 1A). A small-scale freeze-thaw method with modifications provided by J. W. Biddle and J. K. Rasheed was used for comparison of KPC-1 and KPC-2 (3) (Fig. 1B). Detection of carbapenemase activity was performed by bioassay as described previously (24). Analytical isoelectric focusing was performed in polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5; APBiotech, Piscataway, N.J.). β-Lactamase activity was visualized with nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom, or Becton-Dickinson, Lexington, Ky.). In situ inhibition of β-lactamase activity was performed by soaking the gels in solutions containing clavulanate (0.5 mM), tazobactam (0.5 mM), or EDTA (5 mM). Maximum hydrolysis rates for various β-lactam substrates were estimated by UV spectrophotometry, and inhibition by clavulanate and tazobactam was assessed by using nitrocefin as the reporter substrate. The respective procedures were as described previously (5).

Nucleotide sequence accession number. The sequence of the 5.2-kb fragment of pST-H1 containing bla_{KPC-2} has been submitted to the GenBank database and assigned accession number AF481906.

RESULTS

Salmonella serotype Cubana 4707 exhibited either resistance or decreased susceptibility to all β-lactams tested including β-lactam–β-lactamase inhibitor combinations, oxyimino-cephalosporins, aztreonam, and carbapenems. In the presence of clavulanic acid, the MICs of ceftazidime and imipenem were decreased by 4 and 2 doubling dilutions, respectively (Table 1). The isolate was also resistant to streptomycin, trimethoprim, and sulfamethoxazole. The isolate was susceptible to nalidixic acid (MIC, ≤4 μg/ml) and ciprofloxacin (MIC, ≤0.015 μg/ml).

* Corresponding author. Mailing address: National Center for Infectious Diseases, MS G29, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333. Phone: (404) 639-2000. Fax: (404) 639-4290. E-mail: zyr3@cdc.gov.

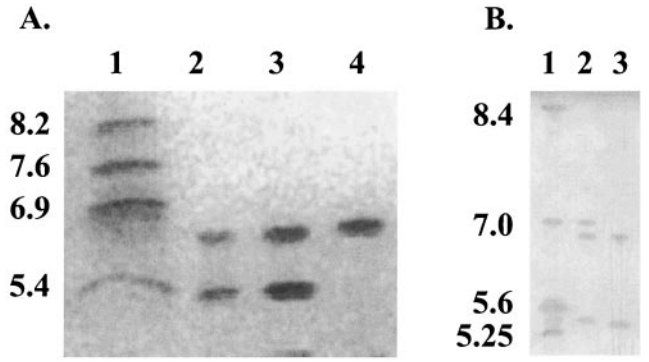

FIG. 1. (A) Isoelectric focusing of β-lactamase preparations from *Salmonella* serotype Cubana 4707, *E. coli*(pST4707), and *E. coli*(pST-H1) (lanes 2, 3, and 4, respectively). β-Lactamases with known pIs are in lane 1 (TEM-1, pI 5.4; PSE-3, pI 6.9; SHV-1, pI 7.6; SHV-5, pI 8.2). (B) Comparison of preparations from *K. pneumoniae* 1534 (lane 2) and *Salmonella* serotype Cubana 4707 (lane 3). β-Lactamases with known pIs are in lane 1 (TEM-12, pI 5.25; TEM-2, pI 5.6; SHV-3, pI 7.0; MIR-1, pI 8.4).

TABLE 1. Susceptibilities to antibiotics of *Salmonella* serotype Cubana 4707, an *E. coli* transconjugant containing wild-type plasmid pST4707, and an *E. coli* DH5α transconjugant containing plasmid pSTH-1

Antibiotic	MIC (μg/ml)			
	Serotype Cubana 4707	*E. coli*(pST4707)	*E. coli* DH5α(pSTH-1)	*E. coli* DH5α
Ampicillin	>256	>256	>256	2
Amoxicillin	>256	>256	>256	4
Amoxicillin-CLA[a]	64	32	8	2
Ticarcillin	>128	>128	>128	0.5
Ticarcillin-CLA[b]	>128	>128	>128	0.5
Piperacillin	>128	>128	>128	1
Piperacillin-TAZ[c]	128	>128	32	1
Cefamandole	>64	>64	>64	1
Cefoxitin	8	16	8	2
Ceftazidime	64	32	8	≤0.5
Ceftazidime-CLA[c]	4	2	2	≤0.5
Cefotaxime	32	16	8	≤0.5
Ceftriaxone	64	64	16	≤0.5
Cefepime	8	8	4	≤0.5
Aztreonam	64	64	32	≤0.5
Imipenem	16	16	8	≤0.5
Imipenem-CLA[c]	4	2	1	≤0.5
Meropenem	8	8	2	≤0.5

[a] CLA, clavulanic acid. Penicillin/inhibitor ratio, 2:1.
[b] The inhibitor concentration was fixed at 2 μg/ml.
[c] TAZ, tazobactam. The inhibitor concentration was fixed at 4 μg/ml.

Transfer of β-lactam resistance to *E. coli* by conjugation was successful. Transconjugants exhibited a phenotype of resistance to β-lactams similar to that of *Salmonella* serotype Cubana 4707 (Table 1). They were also resistant to streptomycin, trimethoprim, and sulfonamides. Analysis of plasmid DNA indicated transfer of a plasmid (pST4707) that, according to conventional gel electrophoresis in a 0.8% gel, was between 24.5 and 42 MDa (data not shown).

β-Lactamase extracts from *Salmonella* serotype Cubana 4707 and *E. coli*(pST4707) were positive in a carbapenemase bioassay performed as described by Yigit et al. (24; data not shown). Isoelectric focusing demonstrated that the extracts contained two β-lactamases with apparent pIs of 5.4 and 6.7, respectively (Fig. 1). Both enzymes were inhibited in situ by clavulanic acid and tazobactam but not by EDTA. The β-lactamase with a pI of 5.4 is consistent with a TEM-1 enzyme, the presence of which was supported by sequencing of 95% of the $bla_{\text{TEM-1}}$-coding region (data not shown).

Cloning of a 5.2-kb *Hin*dIII fragment of pST4707 yielded a pBCSK(+) derivative (pST-H1) which mediated resistance only to β-lactams. *E. coli*(pST-H1) exhibited resistance or decreased susceptibility to all β-lactam antibiotics tested, including carbapenems (Table 1). Also, it was positive in the carbapenemase bioassay and produced a single β-lactamase with a pI of 6.7 (Fig. 1). Hydrolysis experiments performed with extracts from *E. coli*(pST-H1) showed that the rate of imipenem hydrolysis relative to that of penicillin G, which was set at 100, was 45 ± 5. The value for cefotaxime was 58 ± 3. Hydrolysis rates for ceftazidime and cefoxitin were too low to obtain reliable values, although the slightly elevated cefoxitin MIC indicates some degree of hydrolysis (Table 1). The hydrolysis rates for nitrocefin and cephalothin were significantly higher than that for penicillin G (2.4- and 3.1-fold, respectively). The 50% inhibitory concentrations of clavulanate and tazobactam were 1.4 and 0.08 μM, respectively.

The 5.2-kb *Hin*dIII fragment included an 882-bp open reading frame (ORF) that differed by only 1 bp (nucleotide 520) from $bla_{\text{KPC-1}}$ found in *Klebsiella pneumoniae* (24). An identi-

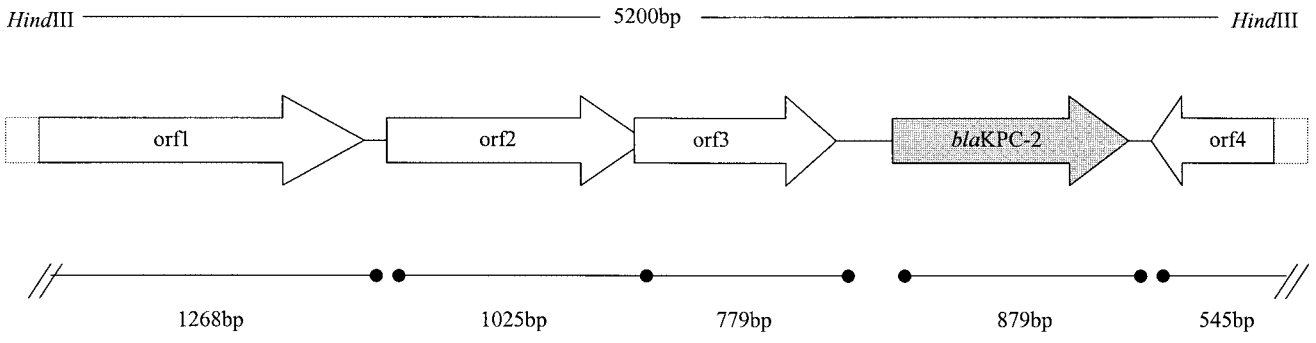

FIG. 2. Schematic representation of the cloned *Hin*dIII fragment (GenBank accession number AF481906). Note that an additional *Hin*dIII site is located within the $bla_{\text{KPC-2}}$ gene.

TABLE 2. ORFs, including bla_{KPC-2}, of the 5.2-kb *Hin*dIII fragment of plasmid pST4707

ORF	Length (no. of amino acids)	Position	G + C content (%)	BLASTP scores (% identity-% similarity)	GenBank accession no.	Remarks
orf1	422	1–1269	60.5	1833 (83–89)	T28654	Transposase from *P. putida*
orf2	341	1376–2401	63.6	829 (55–68)	AAL47163	Putative transposase from *R. solanacearum*
orf3	259	2398–3177	61.9	931 (72–85)	AAL47164	Putative transposition helper protein from *R. solanacearum*
bla_{KPC-2}	293	3564–4445	61.7	Identical	AY034847	Class A carbapenemase KPC-2
orf4	177	5228–4964	61.2	259 (35–52)	AAL50017	Putative transposase from *Ralstonia metalidurans*

cal sequence (bla_{KPC-2}), also from *K. pneumoniae*, has recently appeared in GenBank (accession number AY034847; Moland et al., 41st ICAAC). The deduced protein, KPC-2, differs from KPC-1 by one amino acid residue (KPC-2 contains Gly instead of Ser at position 175). Gly-175 is also found in SME-1, NMC-A, and IMI-1 (9, 12, 16). KPC-2 contained the motifs typical for class A β-lactamases (7) as well as residues characteristic for the carbapenem-hydrolyzing enzymes of this molecular class, including Cys at positions 69 and 238, His at position 105, and a hydroxylated residue (Thr) at position 237 (14, 15, 18). The putative secretory signal sequence comprises 24 residues. The mature KPC-2 has a predicted molecular weight of 28,480. The calculated pI (6.4) differs slightly from the apparent pI of the native β-lactamase. The pI reported for KPC-1 was 6.7 (24). When isoelectric focusing was done with both β-lactamases, the pI of *K. pneumoniae* KPC-1 was very similar to that of KPC-2 from *Salmonella* serotype Cubana (Fig. 1).

The flanking sequences of bla_{KPC-2} (120 bp upstream, including the ribosome binding site and the −10 and −35 regions, and 300 bp downstream) were identical to those reported for bla_{KPC-1} (24). Analysis of the rest of the sequence of the 5.2-kb fragment showed at least four additional ORFs (*orf1* to *orf4*) (Fig. 2). The respective putative peptides (P1 through P4) were significantly similar to proteins associated with transposable elements (Table 2). P1 was highly similar to the C terminus of a transposase A (TnpA) found in the Tn*3* family of transposons from *Pseudomonas putida* (6). P2 and P3 displayed similarity to a TnpA and a transposition helper protein, respectively, found in *Ralstonia solanacearum*. P4 shared a moderate degree of similarity with putative transposases described in *Burkholderia cepacia* and *Ralstonia* spp.

DISCUSSION

KPC-2, along with KPC-1 (24), SME-1 (9), NMC-A (12), and IMI-1 (16), comprise a small group of class A β-lactamases (functional group 2f [2, 15]) with potent carbapenemase activities. The KPC enzymes seem to differ from the rest of the group in that they hydrolyze oxyimino-cephalosporins more efficiently. KPC-2 conferred levels of resistance to β-lactams that were comparable to those conferred by KPC-1 (24). The two enzymes, however, were expressed in different systems, and they also differed by an amino acid residue of the putative omega loop. The latter structure is significant in determining the catalytic properties of class A β-lactamases (7). Hence, differences in the substrate specificity cannot be ruled out.

The high degree of homology between bla_{KPC-1} and bla_{KPC-2} and the identity of their flanking sequences indicate that these variant *bla* genes could be parts of a single structure. However, KPC-2-encoding plasmid pST4707 was self-transferable, while the bla_{KPC-1}-encoding plasmid could not be conjugated into *E. coli* (24). Also, the bla_{KPC-1}-encoding plasmid, unlike pST4707, neither encoded TEM nor mediated resistance to antibiotics other than β-lactams. Therefore, it can be speculated that the bla_{KPC} variants resulted from insertion of a similar or common mobile element into different plasmid backgrounds. Sequencing data are compatible with this notion, although the existence of a transposable element was not proven.

KPC-1 was found in a *K. pneumoniae* isolate from a hospital in North Carolina. KPC-producing *K. pneumoniae* strains have also been found in a Maryland hospital (Moland et al., 41st ICAAC). The isolation of *Salmonella* serotype Cubana 4707, also in Maryland, indicates a certain degree of spread of bla_{KPC} genes in the United States. The plasmid locations of these genes and their association with possibly mobile structures may facilitate their spread. Continued surveillance is essential to monitor for the potential spread of bla_{KPC} genes among *Salmonella* strains.

ACKNOWLEDGMENTS

We thank Jennifer Nelson, David Blythe, Dipti Shah, and David I. Wheeler for contributions to this work.

REFERENCES

1. **Bradford, P. A., Y. Yang, D. Sahm, I. Grop, D. Gardovska, and G. Storch.** 1998. CTX-M5, a novel cefotaxime hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob. Agents Chemother. **42:**1980–1984.
2. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
3. **Bush, K., and S. B. Singer.** 1989. Effective cooling allows sonication to be used for liberation of β-lactamases from gram-negative bacteria. J. Antimicrob. Chemother. **24:**82–84.
4. **Fey, P., P. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs.** 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. N. Engl. J. Med. **342:** 1242–1249.
5. **Giakkoupi, P., L. S. Tzouvelekis, A. Tsakris, V. Loukova, D. Sofianou, and E. Tzelepi.** 2000. IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. Antimicrob. Agents Chemother. **44:**2247–2253.
6. **Lauf, U., C. Muller, and H. Herrmann.** 1998. The transposable elements resident on the plasmids of *Pseudomonas putida* strain H, Tn*5501* and Tn*5502*, are cryptic transposons of the Tn*3* family. Mol. Gen. Genet. **259:** 674–678.
7. **Matagne, A., J. Lamotte-Brasseur, and J.-M. Frere.** 1998. Catalytic properties of class A β-lactamases: efficiency and diversity. Biochem. J. **330:**581–598.
8. **Morosini, M. I., R. Canton, J. Martinez-Beltran, M. C. Negri, J. C. Perez-**

Diaz, F. Baquero, and J. Blazquez. 1995. New extended-spectrum TEM-type β-lactamase from *Salmonella enterica* subsp. *enterica* isolated in a nosocomial outbreak. Antimicrob. Agents Chemother. **39:**458–461.

9. **Naas, T., L. Wandel, W. Sougakoff, D. M. Livermore, and P. Nordmann.** 1994. Cloning and sequence analysis of the gene for a carbapenem-hydrolyzing class A β-lactamase, Sme-1, from *Serratia marcescens* S6. Antimicrob. Agents Chemother. **38:**1262–1270.
10. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved standard M7-A4 (M100-S7). National Committee for Clinical Laboratory Standards, Wayne, Pa.
11. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests, 6th ed. Approved standard M2-A6 (M100-S7). National Committee for Clinical Laboratory Standards, Wayne, Pa.
12. **Nordmann, P., S. Mariotte, T. Naas, R. Labia, and M.-H. Nicolas.** 1993. Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*. Antimicrob. Agents Chemother. **37:**939–946.
13. **Poupart, M. C., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1991. Identification of CTX-2, a novel cefotaximase from a *Salmonella mbandaka* isolate. Antimicrob. Agents Chemother. **35:**1498–1500.
14. **Raquet, X., J. Lamotte-Brasseur, F. Bouillenne, and J.-M. Frere.** 1997. A disulfide bridge near the active site of carbapenem-hydrolyzing class A β-lactamases might explain their unusual substrate profile. Proteins **27:**47–58.
15. **Rasmussen, B. A., and K. Bush.** 1997. Carbapenem-hydrolyzing β-lactamases. Antimicrob. Agents Chemother. **41:**223–232.
16. **Rasmussen, B. A., K. Bush, D. Keeney, Y. Yang, R. Hare, C. O'Gara, and A. A. Medeiros.** 1996. Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **40:**2080–2086.
17. **Revathi, G., K. P. Shannon, P. D. Stapleton, B. K. Jain, and G. L. French.** 1998. An outbreak of extended-spectrum beta-lactamase-producing *Salmonella senftenberg* in a burns ward. J. Hosp. Infect. **40:**295–302.
18. **Sougakoff, W., T. Naas, P. Nordmann, E. Collatz, and V. Jarlier.** 1999. Role of Ser-237 in the substrate specificity of the carbapenem-hydrolyzing class A β-lactamase Sme-1. Biochim. Biophys. Acta **1433:**153–158.
19. **Tassios, P. T., M. Gazouli, E. Tzelepi, H. Milch, N. Kozlova, S. V. Sidorenko, N. J. Legakis, and L. S. Tzouvelekis.** 1999. Spread of a *Salmonella typhimurium* clone resistant to expanded-spectrum cephalosporins in three European countries. J. Clin. Microbiol. **37:**3774–3777.
20. **Vahaboglu, H., L. M. Hall, L. Mulazimoglu, S. Dodanli, I. Yildirim, and D. M. Livermore.** 1995. Resistance to extended-spectrum cephalosporins, caused by PER-1 beta-lactamase, in *Salmonella typhimurium* from Istanbul, Turkey. J. Med. Microbiol. **43:**294–299.
21. **Villa, L., C. Pezzella, F. Tosini, P. Visca, A. Petrucca, and A. Carattoli.** 2000. Multiple-antibiotic resistance mediated by structurally related IncL/M plasmids carrying an extended-spectrum β-lactamase gene and a class 1 integron. Antimicrob. Agents Chemother. **44:**2911–2914.
22. **Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffman, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern.** 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC β-lactamase. Antimicrob. Agents Chemother. **44:**2777–2783.
23. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern.** 2001. Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. **45:**2716–2722.
24. **Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-Sanchez, J. W. Biddle, C. D. Steward, S. Alberti, K. Bush, and F. C. Tenover.** 2001. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45:**1151–1161.