than or equal to an eightfold decrease in the MIC of CAZ in combination with APB was indicative of the production of plasmid-mediated class C β-lactamases in *E. coli* and *K. pneumoniae*. Most of the isolates showed more than or equal to an eightfold reduction in the MIC of CAZ in the presence of APB, while only a fourfold reduction of MIC was observed in *E. coli* NCB03522 (Fig. 3). For ACT-1-producing *K. pneumoniae* BronxLebanon 18, this test was positive, with a 16-fold reduction in the MIC of CTX in combination with APB (Fig. 3). As shown in Fig. 4, the classes of β-lactamases produced by clinical isolates can be easily distinguished from each other by using three kinds of inhibitors, especially when a strain chiefly produces a single type of β-lactamase.

Moreover, we applied the former two methods to several CAZ-resistant clinical isolates of *E. cloacae*, *C. freundii*, *S. marcescens*, and *Pseudomonas aeruginosa* for the detection of their chromosomal AmpC β-lactamases. Most of these isolates showed positive results, suggesting that they are probably hyperproducers of chromosomal AmpC β-lactamases. The results of both tests for the representative strains, *E. cloacae* HKY226, *C. freundii* HKY543, *S. marscecens* HKY-S, and *P. aeruginosa* P-492, are shown in Fig. 1D. For the *E. cloacae* isolates, successful detection was achieved by shortening the center-to-center distance of the two disks containing CAZ and APB from 18 mm to 12 mm in DDST. A few isolates of *S. marcescens* and *P. aeruginosa* were less inhibited by APB, so they could not be detected by either method (data not shown). They might produce additional unknown β-lactamases other than the AmpC type or overexpress their multidrug efflux systems (12).

According to these results, all three tests, the disk potentiation test, the double-disk synergy test, and the microdilution test with APB, were very simple, highly sensitive, and specific for the identification of bacteria producing class C β-lactamases. Thus, they are fully applicable for routine use in clinical microbiology laboratories. Although the results for the production of class C enzymes obtained by these methods is sometimes ambiguous when the strains also coproduce a large amount of ESBLs or MBLs, the methods provide useful information on the mechanism of drug resistance mediated by class C β-lactamases for enhanced infection control and effective antimicrobial therapy.

ACKNOWLEDGMENTS

We thank Kumiko Kai for technical assistance. *K. pneumoniae* P20 producing LAT-1 was a kind gift from E. Tzelepi; and *K. pneumoniae* BronxLebanon 18 producing ACT-1, *E. coli* Coral Gables 66040 producing FOX-5, and *E. coli* Coral J53 (a transformant) producing ACT-1 were graciously provided by G. A. Jacoby. *K. pneumoniae* 5064 producing FOX-5 was the kind gift of A. M. Queenan.

This research was supported by grants from the Ministry of Health, Labor and Welfare of Japan (grants Shinkou-H15-09 and Shinkou-H15-10).

REFERENCES

1. **Arakawa, Y., N. Shibata, K Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38**:40–43.
2. **Barnaud, G., G. Arlet, C. Verdet, O. Gaillot, P. H. Lagrange, and A. Philippon.** 1998. *Salmonella enteritidis*: AmpC plasmid-mediated inducible β-lactamase (DHA-1) with an *ampR* gene from *Morganella morganii*. Antimicrob. Agents Chemother. **42**:2352–2358.
3. **Bauernfeind, A., Y. Chong, and S. Schweighart.** 1989. Extended broad-spectrum β-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection **17**:316–321.
4. **Beesley, T., N. Gascoyne, V. Knott-Hunziker, S. Petursson, S. G. Waley, B. Jaurin, and T. Grundstrom.** 1982. The inhibition of class C β-lactamases by boronic acids. Biochem. J. **209**:229–233.
5. **Black, J. A., K. S. Thomson, and J. D. D. Pitout.** 2004. Use of β-lactamase inhibitors in disk tests to detect plasmid-mediated AmpC β-lactamases. J. Clin. Microbiol. **42**:2203–2206.
6. **Bradford, P. A., C. Urban, N. Mariano, S. A. Projan, J. J. Rahal, and K. Bush.** 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob. Agents Chemother. **41**: 563–569.
7. **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.
8. **Buzzoni, V., J. Blazquez, S. Ferrari, S. Calo, A. Venturelli, and M. P. Costi.** 2004. Aza-boronic acids as non-β-lactam inhibitors of AmpC-β-lactamase. Bioorg. Med. Chem. Lett. **14**:3979–3983.
9. **Coudron, P. E., E. S. Moland, and K. S. Thomson.** 2000. Occurrence and detection of AmpC β-lactamases among *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates at a veterans medical center. J. Clin. Microbiol. **38**:1791–1796.
10. **Doi, Y., N. Shibata, K. Shibayama, K. Kamachi, H. Kurokawa, K. Yokoyama, T. Yagi, and Y. Arakawa.** 2002. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **46**:2427–2434.
11. **Doi, Y., J. Wachino, M. Ishiguro, H. Kurokawa, K. Yamane, N. Shibata, K. Shibayama, K. Yokoyama, H. Kato, T. Yagi, and Y. Arakawa.** 2004. Inhibitor-sensitive AmpC β-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob. Agents Chemother. **48**:2652–2658.
12. **Dubois, V., C. Arpin, M. Melon, B. Melon, C. Andre, C. Frigo, and C. Quentin.** 2001. Nosocomial outbreak due to a multiresistant strain of *Pseudomonas aeruginosa* P12: efficacy of cefepime-amikacin therapy and analysis of β-lactam resistance. J. Clin. Microbiol. **39**:2072–2078.
13. **Fey, P. D., T. J. 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. **27**: 1242–1249.
14. **Hansen, N. D.** 2003. AmpC β-lactamases: what do we need to know for the future? J. Antimicrob. Chemother. **52**:2–4.
15. **Horii, T., Y. Arakawa, M. Ohta, S. Ichiyama, R. Wacharotayankun, and N. Kato.** 1993. Plasmid-mediated AmpC-type β-lactamase isolated from *Klebsiella pneumoniae* confers resistance to broad-spectrum β-lactams, including moxalactam. Antimicrob. Agents Chemother. **37**:984–990.
16. **Hujer, A. M., M. G. Page, M. S. Helfand, B. Yeiser, and R. A. Bonomo.** 2002. Development of a sensitive and specific enzyme-linked immunosorbent assay for detecting and quantifying CMY-2 and SHV β-lactamases. J. Clin. Microbiol. **40**:1947–1957.
17. **Kurokawa, H., T. Yagi, N. Shibata, K. Shibayama, K. Kamachi, and Y. Arakawa.** 2000. A new SHV-derived extended-spectrum β-lactamase (SHV-24) that hydrolyzes ceftazidime through a single-amino-acid substitution (D179G) in the omega-loop. Antimicrob. Agents Chemother. **44**:1725–1727.
18. **Kurokawa, H., N. Shibata, Y. Doi, K. Shibayama, K. Kamachi, T. Yagi, and Y. Arakawa.** 2003. A new TEM-derived extended-spectrum β-lactamase (TEM-91) with an R164C substitution at the omega-loop confers ceftazidime resistance. Antimicrob. Agents Chemother. **47**:2981–2983.
19. **Liebana, E., M. Gibbs, C. Clouting, L. Barker, F. A. Crifton-Hardley, E. Pleydell, B. Abdalhamid, N. D. Hanson, L. Martin, C. Poppe, and R. H. Davies.** 2004. Characterization of β-lactamases responsible for resistance to extended-spectrum cephalosporins in *Escherichia coli* and *Salmonella enterica* strains from food-producing animals in the United Kingdom. Microb. Drug Resist. **10**:1–9.
20. **Livermore, D., and D. F. J. Brown.** 2001. Detection of β-lactamase-mediated resistance. J. Antimicrob. Chemother. **48**(Suppl. 1):59–64.
21. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8**:557–584.
22. **Manchanda, V., and N. P. Singh.** 2003. Occurrence and detection of AmpC β-lactamases among gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Deli, India. J. Antimicrob. Chemother. **51**:415–418.
23. **Morandi, F., E. Caselli, S. Morandi, P. J. Focia, J. Blazquez, B. K. Shoichet, and F. Prati.** 2003. Nanomolar inhibitors of AmpC β-lactamase. J. Am. Chem. Soc. **125**:685–695.
24. **Nadjar, D., M. Rouveau, C. Verdet, J. Donay, P. H. Herrmann, A. Lagrange, A. Philippon, and G. Arlet.** 2000. Outbreak of *Klebsiella pneumoniae* producing transferable AmpC-type β-lactamase (ACC-1) originating from *Hafnia alvei*. FEMS Microbiol. Lett. **187**:35–40.
25. **Nagano, N., N. Shibata, Y. Saito, Y. Nagano, and Y. Arakawa.** 2003. Noso-

comial outbreak of infection by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type β-lactamase. J. Clin. Microbiol. **41**:5530–5536.
26. **Nasim, K., S. Elsayed, J. D. D. Pitout, J. Conly, D. L. Church, and D. B. Gregson.** 2004. New method for laboratory detection of AmpC β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. J. Clin. Microbiol. **42**:4799–4802.
27. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
28. **National Committee for Clinical Laboratory Standards.** 2003. Performance standards for antimicrobial disk susceptibility tests, 8th ed. Approved standard M2-A8. National Committee for Clinical Laboratory Standards, Wayne, Pa.
29. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial and susceptibility testing: 14th informational supplement (M100-S14). National Committee for Clinical Laboratory Standards, Wayne, Pa.
30. **Papanicolaou, G. A., A. A. Medeiros, and G. A. Jacoby.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and α-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34**:2200–2209.
31. **Perez-Perez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC-type β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40**:2153–2162.
32. **Philippon, A., G. Arlet, G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46**:1–11.
33. **Pitout, J. D., M. D. Reisbig, E. C. Venter, D. L. Church, and N. D. Hanson.** 2003. Modification of the double-disk test for detection of *Enterobacteriaceae* producing extended-spectrum and AmpC β-lactamases. J. Clin. Microbiol. **41**:3933–3935.
34. **Powers, R. A., J. Blazquez, G. Scott Weston, M. Morosini, F. Baquero, and B. K. Shoichet.** 1999. The complexed structure and antimicrobial activity of a non-β-lactam inhibitor of AmpC β-lactamase. Protein Sci. **8**:2330–2337.
35. **Qin, X., S. J. Weissman, M. F. Chesnut, B. Zhang, and L. Shen.** 2004. Kirby-Bauer disk approximation to detect inducible third-generation cephalosporin resistance in Enterobacteriaceae. Ann. Clin. Microbiol. Antimicrob. **3**:13.
36. **Queenan, A. M., S. Jenkins, and K. Bush.** 2001. Cloning and biochemical characterization of FOX-5, an AmpC-type plasmid-encoded β-lactamase from a New York City *Klebsiella pneumoniae* clinical isolate. Antimicrob. Agents Chemother. **45**:3189–3194.
37. **Scott Weston, G., J. Blazquez, F. Baquero, and B. K. Shoichet.** 1998. Structure-based enchancement of boronic acid-based inhibitors of AmpC β-lactamase. J. Med. Chem. **41**:4577–4586.
38. **Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa.** 2003. PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol. **41**:5407–5413.
39. **Thomson, K. S., and C. C. Sanders.** 1992. Detection of extended-spectrum β-lactamases in members of the family *Enterobacteriaceae*: comparison of the double-disk test and three-dimensional tests. Antimicrob. Agents Chemother. **36**:1877–1882.
40. **Tzouvelekis, L. S., E. Tzelepi, A. F. Mentis, and A. Tsakris.** 1993. Identification of a novel plasmid-mediated β-lactamase with chromosomal cephalosporinase characteristics in Klebsiella pneumoniae. J. Antimicrob. Chemother. **31**:645–654.
41. **Usher, K. C., L. C. Blaszczak, G. Scott Weston, B. K. Shoichet, and S. J. Remington.** 1998. Three-dimensional structure of AmpC β-lactamase from *Escherichia coli* bound to a transition-state analogue: possible implications for the oxyanion hypothesis and inhibitor design. Biochemistry **37**:16082–16092.
42. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, H. Ito, and Y. Arakawa.** 2004. Nosocomial spread of ceftazidime-resistant *Klebsiella pneumoniae* strains producing a novel class A β-lactamase, GES-3, in a neonatal intensive care unit in Japan. Antimicrob. Agents Chemother. **48**:1960–1967.
43. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, and Y. Arakawa.** 2004. Molecular characterization of a cephamycin-hydrolyzing and inhibitor-resistant class A β-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. Antimicrob. Agents Chemother. **48**:2905–2910.
44. **Yagi, T., H. Kurokawa, N. Shibata, K. Shibayama, and Y. Arakawa.** 2000. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol. Lett. **184**:112–119.
45. **Yong, D., R. Park, J. H. Yum, K. Lee, E. C. Choi, and Y. Chong.** 2002. Further modification of the Hodge test to screen AmpC β-lactamase (CMY-1)-producing strains of *Escherichia coli* and *Klebsiella pneumoniae*. J. Microbiol. Methods **51**:407–410.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2006, p. 178–184 Vol. 50, No. 1
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.1.178–184.2006

Novel Plasmid-Mediated 16S rRNA Methylase, RmtC, Found in a *Proteus mirabilis* Isolate Demonstrating Extraordinary High-Level Resistance against Various Aminoglycosides

Jun-ichi Wachino,[1,2] Kunikazu Yamane,[1] Keigo Shibayama,[1] Hiroshi Kurokawa,[1] Naohiro Shibata,[1] Satowa Suzuki,[1] Yohei Doi,[1] Kouji Kimura,[1] Yasuyoshi Ike,[2] and Yoshichika Arakawa[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] and Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Gunma[2] Japan

Received 7 May 2005/Returned for modification 31 July 2005/Accepted 28 September 2005

***Proteus mirabilis* ARS68, which demonstrated a very high level of resistance to various aminoglycosides, was isolated in 2003 from an inpatient in Japan. The aminoglycoside resistance of this strain could not be transferred to recipient strains *Escherichia coli* CSH-2 and *E. coli* HB101 by a general conjugation experiment, but *E. coli* DH5α was successfully transformed by electroporation with the plasmid of the parent strain, ARS68, and acquired an unusually high degree of resistance against aminoglycosides. Cloning and sequencing analyses revealed that the presence of a novel 16S rRNA methylase gene, designated *rmtC*, was responsible for resistance in strain ARS68 and its transformant. The G+C content of *rmtC* was 41.1%, and the deduced amino acid sequences of the newly identified 16S rRNA methylase, RmtC, shared a relatively low level of identity (≤29%) to other plasmid-mediated 16S rRNA methylases, RmtA, RmtB, and ArmA, which have also been identified in pathogenic gram-negative bacilli. Also, RmtC shared a low level of identity (≤28%) with the other 16S rRNA methylases found in aminoglycoside-producing actinomycetes. The purified histidine-tagged RmtC clearly showed methyltransferase activity against *E. coli* 16S rRNA in vitro. *rmtC* was located downstream of an IS*Ecp1*-like element containing *tnpA*. Several plasmid-mediated 16S rRNA methylases have been identified in pathogenic gram-negative bacilli belonging to the family *Enterobacteriaceae*, and some of them are dispersing worldwide. The acceleration of aminoglycoside resistance among gram-negative bacilli by producing plasmid-mediated 16S rRNA methylases, such as RmtC, RmtB, and RmtA, may indeed become an actual clinical hazard in the near future.**

Aminoglycosides have been widely used for the treatment of a variety of bacterial infections (9). These agents bind to the A site of the 16S rRNA of prokaryotic 30S ribosomal subunits and subsequently block bacterial growth through interference with protein synthesis (17). On the other hand, bacteria have acquired resistance to aminoglycosides by producing aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases, aminoglycoside nucleotidyltransferases, and aminoglycoside phosphotransferases (17, 24). Moreover, reduction of affinity for the target site within 16S rRNA by nucleic acid point mutations, the excretion of aminoglycosides by the augmented function of efflux systems, and altered membrane permeability, which leads to the reduced penetration of these agents, also contribute to the intrinsic clinical resistance of bacteria (3, 17).

Recently, as a new mechanism of resistance against aminoglycosides among clinically important pathogenic bacteria, plasmid-mediated 16S rRNA methylase (RmtA) was first characterized in a clinically isolated *Pseudomonas aeruginosa* strain, strain AR-2. This strain was isolated in 1997 in a Japanese hospital and demonstrated consistent resistance to various clinically important aminoglycosides (29). The total sequence of a large plasmid carrying genes for both CTX-M-3 and 16S rRNA methylase was then submitted to the EMBL/GenBank database (accession no. AF550415) on 18 October 2002 by M. Golebiewski et al., although they did not seem to be aware of the presence of the *armA* gene in the sequence deposited in the database. In 2003, the *armA* gene, found in a clinically isolated *Klebsiella pneumoniae* strain, was reported from France (7). RmtB, which was encoded on a nonconjugative plasmid of a clinically isolated *Serratia marcescens* strain, was also reported from Japan in 2004 (6). At present, the three types of plasmid-mediated 16S rRNA methylases described above have been found in pathogenic gram-negative rods. More recently, nosocomial outbreaks caused by 16S rRNA methylase-producing gram-negative bacteria was reported from Taiwan (28). The further global dissemination of 16S rRNA methylase genes among pathogenic bacilli will be a cause of great concern in the near future, because these genes were mediated by some bacterial site-specific recombination and translocation systems such as a transposon (6, 7, 26).

A *Proteus mirabilis* strain, strain ARS68, which displayed a very high level of resistance to various aminoglycosides, was isolated in 2003 from an inpatient in Japan. A preliminary PCR analysis, however, failed to detect any of the known three plasmid-mediated 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, in this strain. Therefore, it was strongly suspected that the *P. mirabilis* ARS68 strain would have a novel 16S rRNA methylase gene. In the present study, the molecular mecha-

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source
Strains		
P. mirabilis ARS68	Clinical isolate resistant to various aminoglycosides	This study
E. coli DH5α	*supE44 ΔlacU169* (*ϕ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB*$^+$	TOYOBO
E. coli CSH-2	*metB* F$^-$ nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli HB101	*thi-1 hsdS20*(r_B^- m_B^+) *supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rps20* (Str^r) *xyl-5 mtl-1*	A. Ohta, Tokyo University
E. coli BL21(DE3)pLysS	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3) pLysS	Novagen
Plasmids		
pARS68	A natural plasmid carrying *rmtC* of *P. mirabilis* ARS68	This study
pBC-E1	A recombinant plasmid carrying a 7.7-kb EcoRI fragment containing *rmtC*	This study
pBC-KB1	A recombinant plasmid carrying a PCR-amplified fragment containing *rmtC* and its promoter	This study
pBC-Sa1	A recombinant plasmid carrying *aph(3′)*	This study
pGEM-rmtC	A recombinant plasmid carrying PCR-amplified *rmtC* ligated to the pGEM-T vector	This study
pET-His-rmtC	A recombinant plasmid carrying *rmtC* ligated to pET29a(+)	This study
pBCSK+	A cloning vector, chloramphenicol^r	Stratagene
pGEM-T	A cloning vector, ampicillin^r	Promega
pET29a(+)	An expression vector, kanamycin^r	Novagen

[a] r, resistant to the indicated antimicrobial agent.

nism underlying a very high level of resistance against various aminoglycosides found in strain ARS68 was elucidated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. mirabilis* strain ARS68 was isolated in August 2003 from a throat swab of an inpatient admitted to a general hospital in Japan. Biochemical phenotypic identification of this strain was performed with a commercially supplied API 20E system (bioMerieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing. The MICs of antimicrobial agents were determined by the agar dilution method with Mueller-Hinton agar plates, according to the protocol recommended by CLSI (formerly the National Committee for Clinical Laboratory Standards) (18). The following antibiotics were obtained from the indicated sources: amikacin, Bristol Pharmaceuticals K. K., Tokyo, Japan; arbekacin, kanamycin, and streptomycin, Meiji Seika Kaisha, Ltd., Tokyo, Japan; gentamicin and sisomicin, Schering-Plough K. K., Osaka, Japan; isepamicin, Asahi Kasei Corporation, Tokyo, Japan; neomycin, Nippon Kayaku Co., Ltd., Tokyo, Japan; and tobramycin, Shionogi & Co. Ltd., Osaka, Japan.

PCR amplification. The sets of PCR primers and amplification conditions used to detect the three 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, are referred to in our recent study (27).

Transfer of aminoglycoside resistance. Conjugal transfer was performed by using *E. coli* CSH-2 (F$^-$ *metB*, resistant to both nalidixic acid and rifampin) or *E. coli* HB101 (resistant to streptomycin) as a recipient by a filter-mating method. Transconjugants were selected on Luria-Bertani (LB) agar plates containing rifampin (100 μg/ml) and kanamycin (30 μg/ml) or arbekacin (10 μg/ml) when *E. coli* CSH-2 was used as the recipient. Two kinds of streptomycin-containing (50 μg/ml) LB agar plates supplemented with kanamycin (30 μg/ml) or arbekacin (10 μg/ml) were also prepared when *E. coli* HB101 was used as the recipient. The plasmid DNA of *P. mirabilis* ARS68 was prepared by the method of Kado and Liu (14). *E. coli* DH5α was transformed with the plasmids of *P. mirabilis* ARS68

TABLE 2. Results of antibiotic susceptibility testing

Aminoglycoside	MIC (μg/ml)				
	P. mirabilis ARS68(pARS68)	*E. coli* DH5α(pARS68)	*E. coli* DH5α(pBC-E1)	*E. coli* DH5α(pBC-KB1)	*E. coli* DH5α(pBCSK+)
4,6-Substituted deoxystreptamine antimicrobials					
Kanamycin group					
Arbekacin	>1,024	512	512	>1,024	0.25
Amikacin	>1,024	1,024	512	>1,024	0.5
Kanamycin	>1,024	>1,024	>1,024	>1,024	1
Tobramycin	1,024	256	128	512	0.25
Gentamicin group					
Gentamicin	>1,024	256	512	>1,024	0.13
Sisomicin	>1,024	512	256	>1,024	0.13
Isepamicin	>1,024	>1,024	1,024	>1,024	0.13
4,5-Substituted deoxystreptamine antimicrobials					
Neomycin	512	16	0.5	1	0.5
Another aminoglycoside					
Streptomycin	4	2	2	2	2

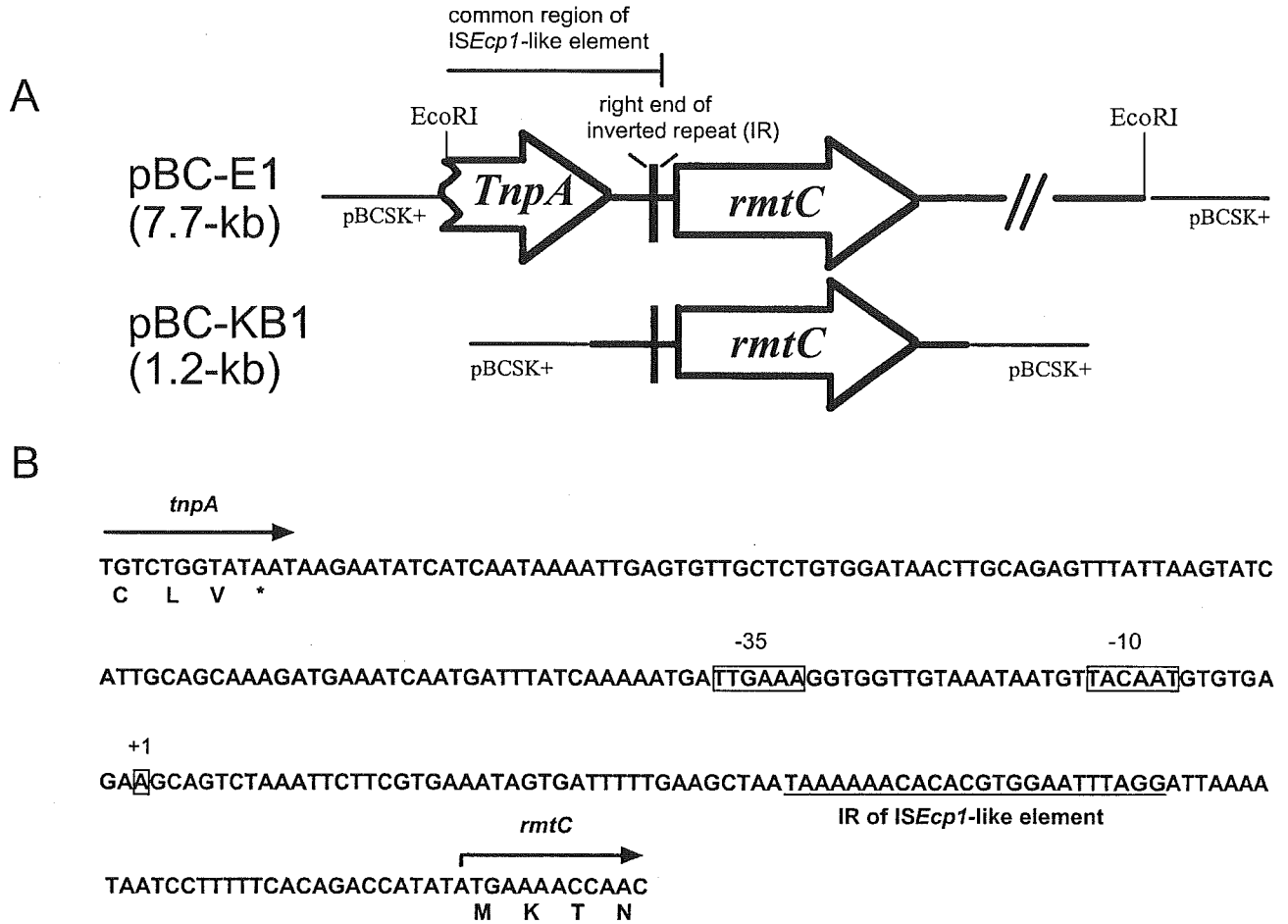

FIG. 1. (A) Schematic presentation of the 7.7-kb EcoRI fragment on pBC-E1 and the 1.2-kb PCR fragment on pBC-KB1. (B) Part of the nucleotide sequences encoding the 3′ end of an IS*Ecp1*-like element and the start region of *rmtC*. The predicted −35 and −10 promoter sequences and the +1 position of the putative transcriptional start of *rmtC* are boxed. These positions were cited elsewhere (4). Arrows indicate the transcription orientation. The deduced amino acid sequences are designated in single-letter code. The right inverted repeat (IR) of an IS*Ecp1*-like element is underlined.

by electroporation techniques. Transformants were selected on LB agar plates supplemented with arbekacin (4 μg/ml) or kanamycin (10 μg/ml).

Cloning and sequencing of aminoglycoside resistance determinants. Both total DNA and plasmid DNA were prepared from the bacterial strains as described previously (23) and restricted with endonucleases according to the recommendations of the supplier. The digested fragments were ligated to restriction enzyme-cleaved pBCSK+ (Stratagene, La Jolla, Calif.), and *E. coli* competent cells were transformed by electroporation with the mixture of recombinant plasmids. Transformants were selected on LB agar plates containing chloramphenicol (30 μg/ml) and arbekacin (4 μg/ml) or kanamycin (10 μg/ml). Both strands of the nucleotide sequences of the cloned fragment encoding the gene responsible for aminoglycoside resistance were determined with BigDye Terminator cycle sequencing ready reaction kits and an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, Calif.) by using several custom sequencing primers.

PCR cloning of aminoglycoside resistance gene. The DNA fragment carrying the aminoglycoside resistance gene and its promoter region was amplified by PCR with the primers rmtC-F (5′-CGC GGA TCC AGT GTA TGA AAA ATG TCT GG-3′) and rmtC-R (5′-CGG GGT ACC GGT GTG TTA GAA TTT GCC TT-3′) (where the underlining indicates the restriction site of BamHI or KpnI). The resultant fragments were digested with BamHI and KpnI and ligated to pBCSK+ (Stratagene).

Expression and purification of histidine-tagged enzyme. The gene responsible for aminoglycoside resistance was amplified from plasmid pBC-E1 by using primers that introduced NdeI and XhoI sites at the ends of the amplified fragments. This fragment was ligated to the pGEM-T vector (Promega, Madison, Wis.), and one plasmid with no amplification error (pGEM-rmtC) was selected. A single nucleotide mutation which leads to the silent mutation (T to C) at position 171 was introduced to destroy the NdeI site within the fragment inserted on pGEM-rmtC by using an LA PCR in vitro mutagenesis kit (Takara Bio Inc., Ohtsu, Japan). A resultant plasmid was digested with NdeI and XhoI and ligated into the pET-29a(+) vector (Novagen, Madison, Wis.) restricted with the same enzymes. The newly constructed expression vector, pET-His-rmtC, was introduced into *E. coli* (DE3)pLysS (Novagen) and cultured in 1 liter of LB broth containing both kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was added when the culture reached an A_{600} of 0.55, and the culture was incubated for an additional 3 h. The bacterial pellet harvested by centrifugation was washed with 50 mM phosphate buffer (pH 7.0) and suspended in 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. The suspension was passed through a French pressure cell (Ohtake Works Co., Ltd., Tokyo, Japan) at 120 MPa and then centrifuged at 100,000 × *g* for 1 h. The supernatant containing the fusion protein was loaded onto a HisTrap HP column and purified according to the manufacturer's instructions (Amersham Biosciences, K. K., Tokyo, Japan). The eluted fusion protein was dialyzed against 20 mM Tris-HCl buffer (pH 7.5), applied to an anion-exchange HiTrap Q HP column (Amersham Biosciences), and eluted with a linear gradient of NaCl. Finally, size-exclusion chromatography was performed with a Superdex 200 HR10/30 column (Amersham Biosciences). The purified protein was dialyzed against HRS buffer (10 mM HEPES-KOH, pH 7.5; 10 mM $MgCl_2$; 50 mM NH_4Cl; 3 mM 2-mercaptoethanol). The purity was checked by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The protein con-

```
RmtC   MKTNDNYIEEVTAKVLTSGKYSTLYPPTVRRVTERLFDRYP-PKQLEKEVRKKLHQAYGA
RmtA   -----MSFDDALASILSSKKYRSLCPDTVRRILDQEWGRHKSPKLAVEATRTRLHGICGA
RmtB   -----MNINDALTSILASKKYRALCPDTVRRILTEEWGRHKSPKQTVEAARTRLHGICGA
ArmA   -----MDKNDVVKKILESKKYENLDSDIVEKVVSISEKKYK-LKEVENYSKKKLHQIWGS
                ::.  .:* * **  * .  *.::      ::   *   :  :.:**   *:

RmtC   YIGGIDGKRLEKKIEKIIHEIPNPTTDEATRTEWEKEICLKILNLHTSTNERTVAYDELY
RmtA   YVT-------------------PESLKAAAAALSVGDVQKALSLHASTKERLAELDCLY
RmtB   YVT-------------------PESLKAAAAALSAGDVKKALSLHASTKERLAELDTLY
ArmA   YYSAYP----------------NWDKLLKKYNQGQLSIEDLLKIHSSTNERVATLNDFY
       *                                        . *.:*:**:** .  : :*

RmtC   QKIFEVTGVPTSITDAGCALNPFSFPFFTEAGMLGQYIGFDLDKGMIEAIEHSLRTLNAP
RmtA   DFIFSG-GVPHRVLDIACGLNPLALFIRDITSVWACDIHQGLGDVITPFAHH-----QGL
RmtB   DFIFSA-ETPRRVLDIACGLNPLALYERGIASVWGCDIHQGLGDVITPFARE-----KDW
ArmA   TYVFGNIKHVSSILDFGCGFNPLALYQWNENEKIIYHAYDIDRAEIAFLSSIIGKLKTTI
         :*        : * .*.:**:::                  :

RmtC   EGIVVKQGDILSDPSGESDLLLMFKLYTLLDRQEEASGLKILQEWKYKNAVISFPIKTIS
RmtA   DFTFALQDVMCTPPTETGDLALVFKLLPLLEREQAGAAMALLQALATPRIAVSFPTRSLG
RmtB   DFTFALQDVLCAPPAEAGDLALIFKLLPLLEREQAGSAMALLQSLNTPRMAVSFPTRSLG
ArmA   KYRFLNKESDVYKG--TYDVVFLLKMLPVLK-QQDVNILDFLQLFHTQNFVISFPIKSLS
       .  .  :          *: :::*: .:*. ::    : :**     . .:*** :::.

RmtC   GRDVGMEENYTVKFENDLVGSDLRIMQKLKLGNEMYFIVSRL--
RmtA   GRGKGMEANYSAWFEGALP-DEFEIEDTKTIGIELVYMIKRNK-
RmtB   GRGKGMEANYAAWFEGGLP-AEFEIEDKKTIGTELIYLIKKNG-
ArmA   GKEKGMEENYQLWFESFTK-GWIKILDSKVIGNELVYITSGFQK
       *:  *** **   **.      :.* :.  :* *: :: .
```

FIG. 2. Alignment of the deduced amino acid sequence of RmtC with those of RmtA, RmtB, and ArmA. Asterisks indicate the conserved residues among the above four 16S rRNA methylases.

centration was estimated by use of the Coomassie Plus protein assay reagent and bovine serum albumin as a standard (Pierce Biotechnology, Rockford, Ill.). The N-terminal sequence of the purified protein was obtained by Edman degradation in a Shimadzu model PPSQ-23 automated protein sequencer.

Preparation of 30S ribosomal subunits. The 30S ribosomal subunits of *E. coli* DH5α were prepared as described by Skeggs et al. (25). After ultracentrifugation with sucrose density gradients, fractions of the 30S ribosomal subunits were collected and concentrated by centrifugation with an Ultrafree-15 centrifugal filter device (Millipore Corporation, Bedford, Mass.). The purity of the 30S ribosomal subunit was checked by denatured agarose gel electrophoresis of the 16S rRNA derived from the material, and the 30S ribosomal subunit was stored at −80°C in aliquots until use.

Methylation assay of 30S ribosomal subunits. The methylation assay of the 30S ribosomal subunits was carried out as described by Doi et al. (6), with some modifications, as follows. The reaction mixture contained 20 pmol 30S ribosomal subunits from *E. coli* DH5α, 20 pmol histidine-tagged RmtC, and 5 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]SAM); and this mixture was adjusted to 200 μl with methylation buffer (50 mM HEPES-KOH, pH 7.5; 7.5 mM $MgCl_2$; 37.5 mM NH_4Cl; 3 mM 2-mercaptoethanol). In control experiments, histidine-tagged RmtC was replaced by an equal volume of heat-inactivated histidine-tagged RmtC, bovine serum albumin, and HRS buffer. Samples (35 μl) were taken at 0, 5, 15, 30, and 60 min and purified with an RNeasy Mini kit (QIAGEN K. K., Tokyo, Japan), according to the instructions provided by the manufacturer. Two micrograms of eluted 16S rRNA was spotted onto a DEAE filter mat for MicroBeta (Perkin-Elmer Life Sciences Japan Co., Ltd., Tokyo, Japan). The filter mat was then covered with MeltiLex for MicroBeta filters (Perkin-Elmer) on a hot plate. Finally, it was applied to a 1450 MicroBeta TRILUX (Perkin-Elmer), and the radioactivity of each spot was counted.

Nucleotide sequence accession number. The open reading frame of *rmtC* was deposited in the EMBL and GenBank databases through the DDBJ database and has been assigned accession number AB194779.

RESULTS

Characteristics of *P. mirabilis* strain ARS68. Clinically isolated *P. mirabilis* strain ARS68 showed an extraordinary high level of resistance (MIC, ≥1,024 μg/ml) to the various clinically important aminoglycosides except streptomycin and neomycin, as shown in Table 2. PCR analyses were performed preliminarily to detect three 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, which were previously found in pathogenic gram-negative bacilli; but none of them was detected in this strain.

Transfer of aminoglycoside resistance. The aminoglycoside resistance of *P. mirabilis* strain ARS68 could not be transferred to the recipients *E. coli* CSH-2 and *E. coli* HB101 by conjugation under the experimental conditions used in this study. However, *E. coli* DH5α was successfully transformed by electroporation with the plasmid, pARS68, prepared from *P. mirabilis* ARS68. The size of plasmid pARS68 was estimated to be ca. >100 kb by summation of the SacI-digested DNA fragment sizes observed by agarose gel electrophoresis (data not shown).

TABLE 3. Amino acid identities among various 16S rRNA methylases

16S rRNA methylase	G+C content (%)	Identity (%) of amino acid residues							
		Plasmid-mediated 16S rRNA methylases among pathogenic gram-negative bacilli			Chromosomally encoded 16S rRNA methylases among aminoglycoside-producing actinomycetes				
		RmtA	RmtB	ArmA	GrmA	KgmB	GrmO	FmrO	Kmr
RmtC	41.1	27.7	29.5	27.8	26.5	23.1	25.4	23.0	22.0
RmtA	55.4		82.0	29.2	31.7	29.5	28.1	27.3	28.7
RmtB	55.6			28.9	31.7	26.4	28.9	28.5	26.3
ArmA	30.4				26.3	26.6	20.6	28.0	24.4

E. coli DH5α(pARS68) demonstrated a very high degree of resistance to various aminoglycosides, as was observed in the parent strain (Table 2).

Cloning of aminoglycoside resistance determinant. A cloning experiment was performed to confirm the genetic aminoglycoside resistance determinant of *P. mirabilis* ARS68 and its transformant, *E. coli* DH5α(pARS68). As a result, one recombinant plasmid (pBC-E1) with a 7.7-kb EcoRI insert derived from pARS68 was obtained by selection with arbekacin and chloramphenicol, and the insert was then sequenced. A part of the cloned fragment sequenced is shown in Fig. 1A. The first 0.5 kb of the insert contained the 3′ end of the *tnpA* gene with a terminal inverted repeat (IR). This region containing the IR had a high degree of similarity at the nucleotide level with the IS*Ecp1* element, which was often identified upstream of several genes encoding CTX-M-type β-lactamases and CMY-type cephalosporinases (2, 4, 10, 20, 21). One open reading frame, which encoded 281 amino acids, was located downstream of *tnpA*. A BLAST analysis of the deduced amino acid sequence revealed that the gene product exhibited low-level identities to the 16S rRNA methylases, RmtA, RmtB, and ArmA (28%, 29%, and 28%, respectively), found in pathogenic gram-negative bacilli. The predicted enzyme was designated RmtC, and a comparison of the deduced amino acid sequences of RmtA, RmtB, and ArmA is shown in Fig. 2. RmtC also has a low degree of similarity (≤28%) to other 16S rRNA methylases found in aminoglycoside-producing *Streptomyces* and *Micromonospora* species. The amino acid similarities among 16S rRNA methylases are summarized in Table 3. The putative promoter region of *rmtC* appeared to be located within the IS*Ecp1*-like element, just upstream of the IR generally found among several CTX-M-type and CMY-type β-lactamase genes (Fig. 1B) (4, 10, 20, 22). One Sau3AI fragment carrying the aminoglycoside phosphotransferase gene, *aph(3′)*, was also cloned from *P. mirabilis* strain ARS68 when kanamycin was used as a selection marker.

Antibiotic susceptibilities. The MICs of the aminoglycosides for parental strain *P. mirabilis* ARS68, *E. coli* DH5α(pARS68), *E. coli* DH5α(pBC-E1), and *E. coli* DH5α(pBC-KB1) are shown in Table 2. *E. coli* DH5α(pARS68) demonstrated resistance to all the various aminoglycosides except streptomycin and neomycin. RmtC-producing strains *E. coli* DH5α(pBC-E1) and *E. coli*(pBC-KB1) showed high levels of resistance to 4,6-disubstituted deoxystreptamine antimicrobials belonging to the kanamycin and gentamicin groups but were susceptible to the 4,5-disubstituted deoxystreptamine antimicrobial neomycin and another aminoglycoside, streptomycin. *E. coli* DH5α(pBC-Sa1), which carried the aminoglycoside phosphotransferase gene, *aph(3′)*, showed resis-

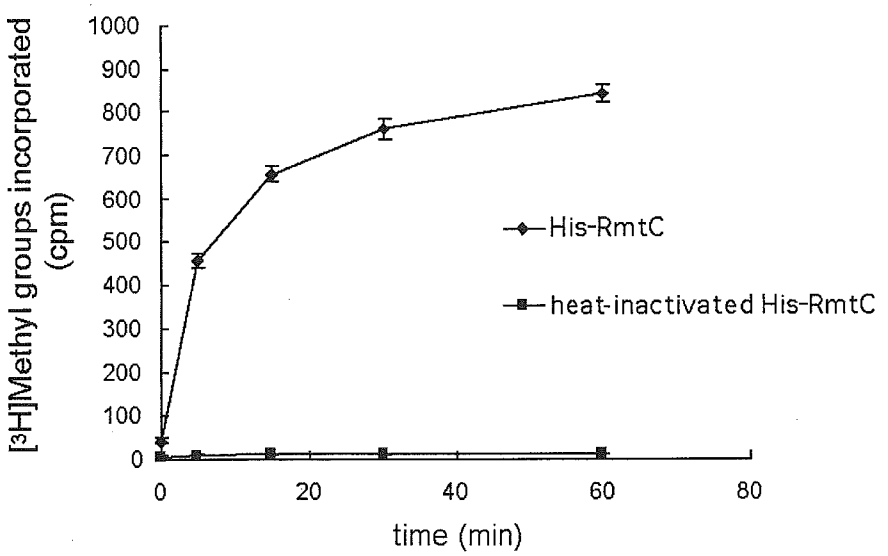

FIG. 3. Methylation of 16S rRNA. The 16S rRNA from *E. coli* DH5α was incubated with purified histidine-tagged RmtC (His-RmtC) by using [*methyl*-^{3}H]SAM as a cofactor. The value of each point was calculated with three datum points. Error bars indicate standard deviations.

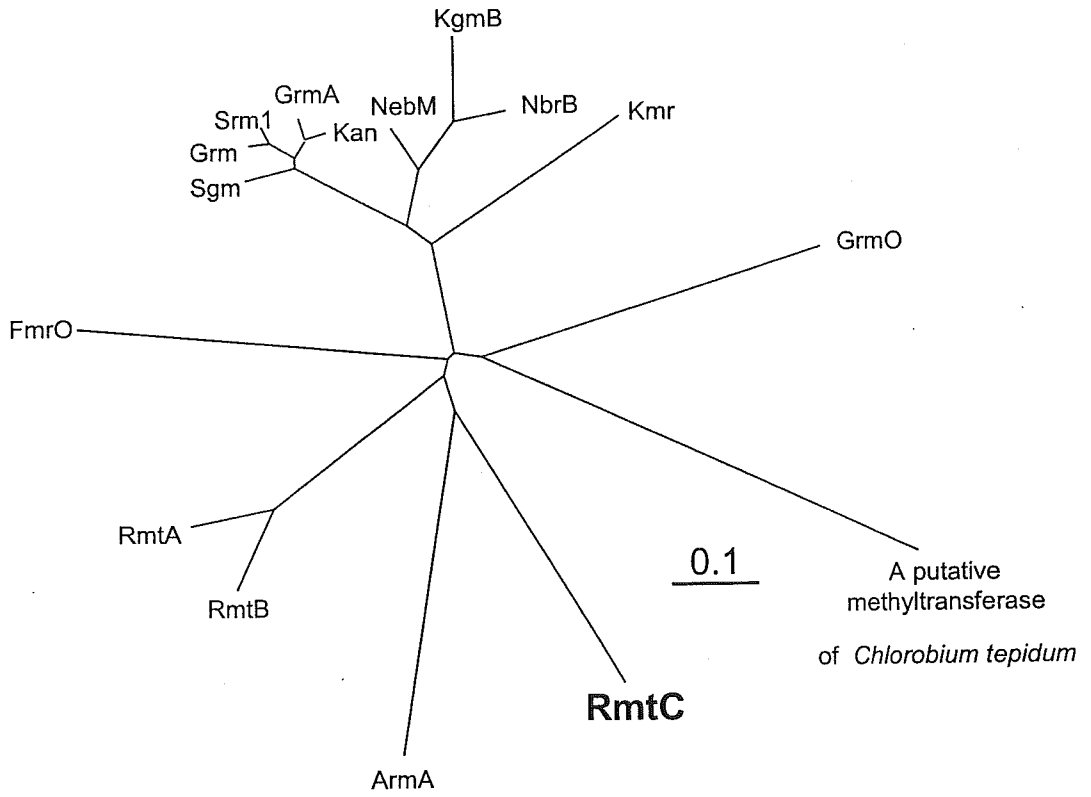

FIG. 4. Dendrogram of 16S rRNA methylases. Sequences are from *P. mirabilis* (RmtC; GenBank accession number AB194779), *P. aeruginosa* (RmtA; GenBank accession number AB083212) (29), *S. marcescens* (RmtB; GenBank accession number AB103506) (6), *K. pneumoniae* (ArmA; GenBank accession number AY220558) (7), *Micromonospora zionensis* (Sgm; GenBank accession number A45282) (16), *Micromonospora rosea* (Grm; GenBank accession number M55521) (15), *Micromonospora inyoensis* (Srm1; GenBank accession number AY661430), *Micromonospora echinospora* (GrmA; GenBank accession number AY524043), *Streptomyces* sp. (Kan; GenBank accession number AJ414669), *Streptomyces tenebrarius* (NebM; GenBank accession number AJ550991), *S. tenebrarius* (KgmB; GenBank accession number S60108) (13), *Streptoalloteichus hindustanus* (NbrB; GenBank accession number AF038408), *Streptoalloteichus kanamyceticus* (Kmr; GenBank accession number AJ582817) (5), *Micromonospora olivasterospora* (FmrO; GenBank accession number D13171) (19), *M. echinospora* (GrmO; GenBank accession number AY524043), and *Chlorobium tepidum* TLS (putative methytransferase; GenBank accession number AAM72273). The "0.1" scale represents a genetic unit reflecting 10% of the amino acid substitutions calculated with the ClustalW program (http://www.ddbj.nig.ac.jp/search/Welcome-e.html) provided by the DDBJ (http://www.ddbj.nig.ac.jp/Welcome-e.html).

tance to both neomycin (MIC, 1,024 μg/ml) and kanamycin (MIC, >1,024 μg/ml). The resistance to neomycin found in strain ARS68 seemed to be attributable to the presence of *aph(3')*.

Identification of RmtC as a 16S rRNA methyltransferase. Histidine-tagged RmtC-producing *E. coli* BL21(DE3)pLysS showed resistance to arbekacin, while *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS, which carried the pET29a(+) vector, were susceptible to arbekacin. This finding indicated that the production of histidine-tagged RmtC was responsible for the aminoglycoside resistance in *E. coli* BL21(DE3)pLysS. The N-terminal sequence of the purified protein was determined to be MKTND. The result of the methylation assay is shown in Fig. 3. Purified histidine-tagged RmtC readily methylated 30S ribosomal subunits prepared from *E. coli* DH5α in the presence of the methyl group donor [*methyl*-^{3}H]SAM as a cosubstrate in a time-dependent manner. On the other hand, incubation with heat-inactivated histidine-tagged RmtC did not increase the counts of radioactivity. When an equal volume of bovine serum albumin or HRS buffer was used in place of purified histidine-tagged RmtC, no increase in the radioactivity counts was observed (data not shown).

DISCUSSION

In the present study, we found a new 16S rRNA methylase gene, *rmtC*, in a clinical *P. mirabilis* isolate and characterized it precisely. The production of RmtC conferred a high degree of resistance mainly against 4,6-disubstituted deoxystreptamines but not against non-4,6-disubstituted deoxystreptamines, such as streptomycin and neomycin, as did RmtA, RmtB, and ArmA. Although the methylation site in the 16S rRNA has not been clarified yet, it was speculated that G1405 within the A site of 16S rRNA would be methylated by these plasmid-mediated 16S rRNA methylases, since the methylation of G1405 by some 16S rRNA methylases produced by actinomycetes was reported to confer resistance against 4,6-disubstituted deoxystreptamines but not against 4,5-disubstituted deoxystreptamines, such as neomycin (1). RmtC as well as RmtA, RmtB, and ArmA might well confer resistance against 4,6-disubstituted deoxystreptamines through a manner similar to that in aminolycoside-producing actinomycetes. The methylation site in the 16S rRNA introduced by these enzymes will be elucidated in a forthcoming study.

Interestingly, all the plasmid-mediated 16S rRNA methylase

genes found so far were associated with some genes implicated in gene recombination systems. For example, the *rmtA* gene was flanked by a 262-bp sequence called the κγ element that was initially found in Tn*5041* and that was predicted to be a relic of mobile genetic elements (26). The *rmtB* gene was located just downstream of the 3′ end of the insertion sequence of Tn*3* (6). As for the two genes described above, the mode of actual translocation of the fragments containing the 16S rRNA methylase genes has not been elucidated in detail. On the other hand, it was reported that the *armA* gene was mediated by a composite transposon Tn*1548* and was successfully transposed in vitro (8). Although the *rmtC* gene was also associated with a *tnpA* gene encoding a probable transposase, the actual mode of translocation of the regions carrying the *rmtC* gene is unclear. However, it is speculated that the presence of an IS*Ecp1*-like element located upstream of *rmtC* would be responsible for the actual translocation process, because several CTX-M-type β-lactamase genes located downstream of *tnpA* within the IS*Ecp1* element were able to be transposed in vitro (4, 21). Characterization of the genetic environment mediating the *rmtC* gene and the mode of translocation will be undertaken in another study.

As was observed in the phylogenic tree (Fig. 4), a cluster of plasmid-mediated 16S rRNA methylases is antithetical to that of the 16S rRNA methylases from actinomycetes. Although no progenitor of plasmid-mediated 16S rRNA methylases, including RmtA, RmtB, RmtC, and ArmA, has been found to date, these genes might have been derived from unknown environmental aminoglycoside-producing bacteria.

In conclusion, we identified a novel plasmid-mediated 16S rRNA methylase, RmtC, in a clinical *P. mirabilis* isolate that demonstrated an extraordinarily high level of aminoglycoside resistance like actinomycetes. The nosocomial transmission of pathogens that produce plasmid-dependent 16S rRNA methylases has recently been reported from Taiwan (28), and an ArmA-producing *E. coli* isolate was isolated from the feces of a diarrheic pig in Spain in 2002 (11, 12). Special caution should be taken because of the emergence and spread of pathogenic bacteria that have acquired various new antimicrobial resistance genes, including *rmtC*, *rmtB*, and *rmtA*, especially in both clinical and livestock farming environments, where large amounts of antimicrobial agents have routinely been used.

ACKNOWLEDGMENTS

We are grateful to Kumiko Kai and Fusako Yokokawa for technical assistance.

This study was supported by the Ministry of Health, Labor and Welfare, Japan (grants H15-Shinkou-9 and H15-Shinkou-10). The research activity of J. Wachino was supported by a Scholarship for Young Scientists, provided by the Japan Society for the Promotion of Science.

REFERENCES

1. **Beauclerk, A. A., and E. Cundliffe.** 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J. Mol. Biol. **193:**661–671.
2. **Boyd, D. A., S. Tyler, S. Christianson, A. McGeer, M. P. Muller, B. M. Willey, E. Bryce, M. Gardam, P. Nordmann, and M. R. Mulvey.** 2004. Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum β-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrob. Agents Chemother. **48:**3758–3764.
3. **Bryan, L. E.** 1988. General mechanisms of resistance to antibiotics. J. Antimicrob. Chemother. **22**(Suppl. A):1–15.
4. **Cao, V., T. Lambert, and P. Courvalin.** 2002. ColE1-like plasmid pIP843 of *Klebsiella pneumoniae* encoding extended-spectrum β-lactamase CTX-M-17. Antimicrob. Agents Chemother. **46:**1212–1217.
5. **Demydchuk, J., Z. Oliynyk, and V. Fedorenko.** 1998. Analysis of a kanamycin resistance gene (*kmr*) from *Streptomyces kanamyceticus* and a mutant with increased aminoglycoside resistance. J. Basic Microbiol. **38:**231–239.
6. **Doi, Y., K. Yokoyama, K. Yamane, J. Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. **48:**491–496.
7. **Galimand, M., P. Courvalin, and T. Lambert.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. **47:**2565–2571.
8. **Galimand, M., S. Sabtcheva, P. Courvalin, and T. Lambert.** 2005. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrob. Agents Chemother. **49:**2949–2953.
9. **Gilbert, D. N., Jr., R. C. Moellering, and M. A. Sande.** 2003. The Sanford guide to antimicrobial therapy 2003. Antimicrobial Therapy, Inc., Hyde Park, N.Y.
10. **Giles, W. P., A. K. Benson, M. E. Olson, R. W. Hutkins, J. M. Whichard, P. L. Winokur, and P. D. Fey.** 2004. DNA sequence analysis of regions surrounding bla_{CMY-2} from multiple *Salmonella* plasmid backbones. Antimicrob. Agents Chemother. **48:**2845–2852.
11. **González-Zorn, B., A. Catalan, J. A. Escudero, L. Dominguez, T. Teshager, C. Porrero, and M. A. Moreno.** 2005. Genetic basis for dissemination of *armA*. J. Antimicrob. Chemother. **56:**583–585.
12. **González-Zorn, B., T. Teshager, M. Casas, M. C. Porrero, M. A. Moreno, P. Courvalin, and L. Domínguez.** 2005. *armA* and aminoglycoside resistance in *Escherichia coli*. Emerg. Infect. Dis. **11:**954–956.
13. **Holmes, D. J., and E. Cundliffe.** 1991. Analysis of a ribosomal RNA methylase gene from *Streptomyces tenebrarius* which confers resistance to gentamicin. Mol. Gen. Genet. **229:**229–237.
14. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
15. **Kelemen, G. H., E. Cundliffe, and I. Financsek.** 1991. Cloning and characterization of gentamicin-resistance genes from *Micromonospora purpurea* and *Micromonospora rosea*. Gene **98:**53–60.
16. **Kojic, M., L. Topisirovic, and B. Vasiljevic.** 1992. Cloning and characterization of an aminoglycoside resistance determinant from *Micromonospora zionensis*. J. Bacteriol. **174:**7868–7872.
17. **Kotra, L. P., J. Haddad, and S. Mobashery.** 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob. Agents Chemother. **44:**3249–3256.
18. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Document M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Ohta, T., and M. Hasegawa.** 1993. Analysis of the self-defense gene (*fmrO*) of a fortimicin A (astromicin) producer, Micromonospora olivasterospora: comparison with other aminoglycoside-resistance-encoding genes. Gene **127:**63–69.
20. **Poirel, L., J. W. Decousser, and P. Nordmann.** 2003. Insertion sequence IS*Ecp1*B is involved in expression and mobilization of a bla_{CTX-M} β-lactamase gene. Antimicrob. Agents Chemother. **47:**2938–2945.
21. **Poirel, L., M. F. Lartigue, J. W. Decousser, and P. Nordmann.** 2005. IS*Ecp1*B-mediated transposition of bla_{CTX-M} in *Escherichia coli*. Antimicrob. Agents Chemother. **49:**447–450.
22. **Saladin, M., V. T. Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Delisle, A. Philippon, and G. Arlet.** 2002. Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS. Microbiol. Lett. **209:**161–168.
23. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
25. **Skeggs, P. A., J. Thompson, and E. Cundliffe.** 1985. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. Mol. Gen. Genet. **200:**415–421.
26. **Yamane, K., Y. Doi, K. Yokoyama, T. Yagi, H. Kurokawa, N. Shibata, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Genetic environments of the *rmtA* gene in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **48:**2069–2074.
27. **Yamane, K., J. Wachino, Y. Doi, H. Kurokawa, and Y. Arakawa.** 2005. Global spread of multiple-aminoglycoside-resistance genes. Emerg. Infect. Dis. **11:**951–953.
28. **Yan, J. J., J. J. Wu, W. C. Ko, S. H. Tsai, C. L. Chuang, H. M. Wu, Y. J. Lu, and J. D. Li.** 2004. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. J. Antimicrob. Chemother. **54:**1007–1012.
29. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362:**1888–1893.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2006, p. 534–541
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.2.534–541.2006

Vol. 50, No. 2

Horizontal Transfer of bla_{CMY}-Bearing Plasmids among Clinical *Escherichia coli* and *Klebsiella pneumoniae* Isolates and Emergence of Cefepime-Hydrolyzing CMY-19

Jun-ichi Wachino,[1,2] Hiroshi Kurokawa,[1] Satowa Suzuki,[1] Kunikazu Yamane,[1] Naohiro Shibata,[1] Kouji Kimura,[1] Yasuyoshi Ike,[2] and Yoshichika Arakawa[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] and Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Gunma,[2] Japan

Received 30 June 2005/Returned for modification 17 August 2005/Accepted 4 November 2005

Nine *Escherichia coli* and 5 *Klebsiella pneumoniae* clinical isolates resistant to various cephalosporins and cephamycins were identified in a Japanese general hospital between 1995 and 1997. All nine *E. coli* isolates and one *K. pneumoniae* isolate carried bla_{CMY-9}, while the other four *K. pneumoniae* isolates harbored a variant of bla_{CMY-9}, namely, bla_{CMY-19}. The pulsed-field gel electrophoresis patterns of the nine CMY-9-producing *E. coli* isolates were almost identical, suggesting their clonal relatedness, while those of the five *K. pneumoniae* isolates were divergent. Plasmid profiles, Southern hybridization, and conjugation assays revealed that the genes for the CMY-9 and the CMY-19 β-lactamases were located on very similar conjugative plasmids in *E. coli* and *K. pneumoniae*. The genetic environment of bla_{CMY-19} was identical to that of bla_{CMY-9}. A single amino acid substitution, I292S, adjacent to the H-10 helix region was observed between CMY-9 and CMY-19. This substitution was suggested to be responsible for the expansion of the hydrolyzing activity against several broad-spectrum cephalosporins, and this finding was consistent with the kinetic parameters determined with purified enzymes. These findings suggest that the bla_{CMY-19} genes found in the four *K. pneumoniae* isolates might have originated from bla_{CMY-9} gene following a point mutation and dispersed among genetically different *K. pneumoniae* isolates via a large transferable plasmid.

Resistance to β-lactam antibiotics in gram-negative bacilli is mainly mediated by the production of β-lactamases, which are divided into four major molecular classes, classes A, B, C, and D (1, 10). Genes for AmpC (class C) β-lactamases are generally encoded on the chromosomes in many gram-negative microbes, including *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Pseudomonas aeruginosa* (27). Chromosomal AmpC enzymes are usually inducible and are often responsible for resistance to cephalosporins (27) as well as to penicillins. Plasmid-mediated class C β-lactamases have mainly been described in *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. throughout the world (25). A cephamycin-resistant *Klebsiella pneumoniae* strain producing a plasmid-mediated class C β-lactamase, CMY-1, was first reported in 1989 in Korea (7, 8). Plasmid-mediated class C enzymes are currently divided into at least five clusters (25) on the basis of amino acid sequence similarities, together with their putative progenitor chromosomal AmpC enzymes. In Japan, MOX-1 (16), CMY-8 (unpublished data), CMY-9 (12), CMY-2 (unpublished data), CFE-1 (23), and DHA-1 (unpublished data) have so far been found as plasmid-mediated AmpC β-lactamases, mainly in nosocomial isolates of the family *Enterobacteriaceae*.

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

Between 1995 and 1997, eight additional *E. coli* isolates and five *K. pneumoniae* isolates resistant to both oximino-cephalosporins and cephamycins were isolated in the same hospital where the first CMY-9-producing *E. coli* strain (strain HKYM68) was isolated in 1995 (12). In the present study, the molecular and biochemical mechanisms underlying the multiple-cephalosporin resistance among these 14 isolates as well as their genetic relatedness were elucidated.

MATERIALS AND METHODS

Bacterial strains. Nine *E. coli* isolates and five *K. pneumoniae* isolates displaying a high level of resistance to cephalosporins and cephamycins were isolated between 1995 and 1997 in a general hospital in Yamaguchi Prefecture, Japan, and stored in our laboratory. Among these isolates, *E. coli* strain HKYM68 was previously found to produce CMY-9 (12). Phenotypic identification of each isolate was performed by using a commercial identification system (API 20E system; bioMerieux, Marcy l'Etoile, France), according to the instructions of the manufacturer.

Phenotypic test for β-lactamase types. A simple initial screening test for the presumptive identification of the β-lactamase types in clinical isolates was performed by use of the double-disk synergy test with Kirby-Bauer disks. Two disks which contained ceftazidime (30 μg per disk) or cefotaxime (30 μg per disk) were used in combination with three different disks containing either amoxicillin-clavulanate (20 μg per disk/10 μg per disk), sodium mercaptoacetic acid (3 mg per disk), or 3-aminophenyl boronic acid (APB) (300 μg per disk), which are specific inhibitors of class A, class B, and class C β-lactamases, respectively (2, 32).

Identification of β-lactamase genes by PCR and sequencing analyses. The samples were screened by PCR with 12 sets of primers for the detection of TEM- and SHV-derived extended-spectrum β-lactamases; GES-type, CTX-M-2-type, CTX-M-3-type, and CTX-M-9-type class A β-lactamases; CMY-1-, CMY-2-, and DHA-1-type class C β-lactamases; and IMP-1-, IMP-2-, and VIM-2-type class B β-lactamases. The sets of PCR primers and the amplification conditions used to detect various plasmid-mediated β-lactamase genes found thus far in Japan have been reported previously (28, 31). The PCR amplicons were electrophoresed on

a 2% agarose gel and purified with a MinElute gel extraction kit (QIAGEN K. K., Tokyo, Japan), and both strands were sequenced.

Transfer of β-lactam resistance. A conjugation experiment was performed by the broth mating method with *E. coli* strain CSH-2 (*metB* F^- Rif^r Nal^r) as the recipient. The donor-to-recipient ratio was 1:4, and the mating time was 3 h. Transconjugants were selected on Luria-Bertani (LB) agar plates supplemented with both rifampin (100 μg/ml) and nalidixic acid (50 μg/ml), together with cefotaxime (10 μg/ml) or ceftazidime (10 μg/ml).

Antibiotic susceptibility tests. Susceptibilities to antibiotics were tested by the agar dilution method according to the procedure recommended by the CLSI (formerly the National Committee for Clinical Laboratory Standards) document M7-A5 (24). *E. coli* ATCC 25922 was used as the control strain for the antimicrobial susceptibility testing.

Isoelectric focusing of β-lactamases. Bacterial cells were grown in 10 ml of LB broth supplemented with cephalothin (50 μg/ml) and were harvested by centrifugation (4,000 × *g* for 15 min) The cell pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer. The pI of β-lactamase was determined as described previously (31).

Pulsed-field gel electrophoresis (PFGE). Total DNA preparations containing both chromosomal and plasmid DNAs were extracted from each isolate and digested overnight with XbaI (New England Biolabs, Beverly, MA) in agarose gel plugs. The digested DNAs were subjected to electrophoresis with a CHEF-DRII drive module (Bio-Rad Laboratory, Hercules, CA), with pulses ranging from 12.5 to 40 s at 6 V/cm for 24 h at 16°C.

Plasmid analysis and Southern hybridization. Large plasmids mediating bla_{CMY} genes were prepared from clinical isolates and their transconjugants according to the procedure described by Kado and Liu (17) and electrophoresed on a 0.8% agarose gel. The plasmid DNAs of the transconjugants were also prepared by using a QIAGEN midi-prep kit (QIAGEN K. K.), digested with SacI, and then transferred to a nylon membrane (Bio-Rad Laboratories). The 999-bp digoxigenin (DIG)-labeled DNA probes were prepared by using a PCR DIG Probe Synthesis kit (Roche Diagnostics, Tokyo, Japan); and the DNA template was prepared from a bla_{MOX}-positive *E. coli* strain HKYM68 (12), together with two PCR primers, primers MOX-F (5′-AAC AAC GAC AAT CCA TCC-3′) and MOX-R (5′-TGT TGA AGA GCA CCT GGC-3′).

PCR and sequencing analyses of flanking regions of bla_{CMY}. To determine the genetic environments of the bla_{CMY} genes, standard PCR amplification experiments and sequencing analyses were performed with an Expand High-Fidelity PCR system (Roche) and several sets of primers, which were designed on the basis of the nucleotide sequences deposited in the EMBL/GenBank/DDBJ databases under accession number AB061794. The resultant PCR products were purified by using a MinElute gel extraction kit (QIAGEN) and were subsequently sequenced with the appropriate primers.

Cloning of bla_{CMY-9} and bla_{CMY-19} for purification of enzymes. To amplify bla_{CMY-9} and bla_{CMY-19}, conjugative plasmids pK209 and pK466 were used as the template DNA, respectively. A highly reliable PCR amplification was performed with primers CMY-S1 (5′-CAG GGC GTG AGG ATA AAG-3′) and CMY-S2 (5′-GGG ACG AGA TAG AGA AAT-3′) by using the Expand High-Fidelity PCR system (Roche). Each amplicon was ligated to the pGEM-T vector (Promega, Madison, WI) and subjected to confirmatory sequencing. Selected plasmids with no amplification error, pGEM-CMY-9 and pGEM-CMY-19, which carry bla_{CMY-9} and bla_{CMY-19}, respectively, were digested with XhoI and EcoRI. The resultant fragments were ligated to pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzymes; and competent cells of *E. coli* strain DH5α [*supE44* Δ*lacU169* (ϕ*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB*$^+$], purchased from TOYOBO, Co., Ltd, Tokyo, Japan, were transformed by electroporation with the mixture of the constructed plasmids.

Purification of CMY-9 and CMY-19 β-lactamases. *E. coli* strain DH5α, which harbored pBC-CMY-9 carrying the bla_{CMY-9} gene or pBC-CMY-19 carrying the bla_{CMY-19} gene, was separately cultured overnight in 2 liters of LB broth containing cephalothin (50 μg/ml) and chloramphenicol (30 μg/ml). The cells were harvested by centrifugation and washed in 50 mM sodium phosphate buffer (pH 7.0). The pellets were resuspended with 10 ml of 20 mM Tris-HCl buffer (pH 7.5) and destroyed with a French press. After low-speed centrifugation (3,300 × *g* for 15 min) to remove the cellular debris and unbroken cells, the supernatant was again centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant containing β-lactamase was chromatographed through a HiTrap Q HP column (Amersham Biosciences) that had been preequilibrated with 20 mM Tris-HCl buffer (pH 7.5). β-Lactamase activity was detected in the flowthrough fraction, which was then dialyzed against 50 mM sodium phosphate buffer (pH 6.0). This partially purified fraction was again applied to a HiTrap SP HP column (Amersham Biosciences) that had been preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The enzymes were eluted with a linear gradient of NaCl in the same buffer. Fractions with β-lactamase activity were dialyzed against 50 mM sodium phosphate buffer (pH 7.0) and condensed by use of an Ultrafree-15 centrifuge filter device (Millipore Corporation, Bedford, MA). The production of CMY-19 was not enough in the *E. coli* transformant, so the following method was used. The bla_{CMY-19} gene was amplified with primers CMY-F2 (5′-CAT ATG CAA CAA CGA CAA TCC ATC C-3′), which has an NdeI linker (underlined), and CMY-R2 (5′-GAA TTC TCA ACC GGC CAA CTG CGC CA-3′), which has an EcoRI linker (underlined), and the Expand High-Fidelity PCR system (Roche). The amplicon was ligated with a pGEM-T vector (Promega), subjected to confirmatory sequencing, and then excised by digestion with NdeI and EcoRI and subcloned into the expression vector pET29a(+) (Novagen, Madison, WI), which was cleaved with the same enzymes. The constructed expression vector, named pET-CMY-19, was introduced into *E. coli* BL21(DE3)pLysS [F^- *ompT hsdS*B ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS], which was obtained from Novagen through TAKARA BIO Inc., Kyoto, Japan. The transformant was cultured in 1 liter of LB broth containing kanamycin (50 μg/ml) and chloramphenicol (30 mg/ml) at 37°C. Isopropyl-β-D-thiogalactopyranoside was added when the culture reached an optical density at 600 nm of 0.55, and the culture was incubated for an additional 6 h at 25°C. CMY-19 was purified by the same methods used for the purification of CMY-9. The purity of the β-lactamases was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining. The purified CMY-9 and CMY-19 β-lactamases were also subjected to isoelectric focusing analysis with an Ampholine PAG plate (Amersham Biosciences) and stained with CBB.

Assay of kinetic parameters. The kinetic parameters of CMY-9 and CMY-19 against various β-lactam substrates were assayed at 30°C in 50 mM sodium phosphate buffer (pH 7.0) by using an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; piperacillin, 232 nm; cephalothin, 262 nm; cephaloridine, 297 nm; ceftizoxime, 257 nm; ceftazidime, 274 nm; cefotaxime, 264 nm; cefpirome, 267 nm; cefepime, 275 nm; cefoxitin, 270 nm; cefmetazole, 259 nm; moxalactam, 274 nm; imipenem, 298 nm. K_m and k_{cat} values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. K_i was determined by the procedure described in our previous study (13), with cephalothin used as a reporter substrate.

Nucleotide sequence accession number. The open reading frame of bla_{CMY-19} was deposited in the EMBL/GenBank databases through DDBJ and assigned accession number AB194410.

RESULTS

Properties of nine *E. coli* and five *K. pneumoniae* clinical isolates. The MICs of six β-lactams for the 14 clinical isolates are shown in Table 1. The *K. pneumoniae* and *E. coli* clinical isolates exhibited resistance to oximino-cephalosporins and cephamycins but were susceptible to carbapenems, although *E. coli* HKYM68 also showed resistance to imipenem. In a double-disk synergy test, no synergistic effect of clavulanic acid on the activities of ceftazidime and cefotaxime was detectable in any of the 14 isolates. A lack of metallo-β-lactamase production was also suggested by the results of the sodium mercaptoacetic acid disk tests. An apparent expansion of the growth inhibitory zone was observed with the 14 clinical isolates only between a disk containing 300 μg of 3-aminophenyl-boronic acid and a disk containing ceftazidime or cefotaxime, suggesting the production of a class C β-lactamase. These findings indicate that the property of resistance to oxyimino-cephalosporins and cephamycins was likely due to the production of a class C β-lactamase.

PCR detection of various β-lactamase genes and sequencing revealed that a *K. pneumoniae* isolate (HKY209) carried bla_{CMY-9}, while the other four *K. pneumoniae* isolates carried bla_{CMY-19}, a variant gene of bla_{CMY-9} (Table 1). A single nucleotide mutation at position 944 was found between bla_{CMY-9} and the newly identified bla_{CMY-19} gene, and this point mutation resulted in the I292S substitution near the H-10 helix domain in CMY-19, as shown in Fig. 1. All nine *E. coli* clinical isolates carried both the bla_{CMY-9} and the bla_{TEM} genes (Table 1).

TABLE 1. MICs for parent strains and their transconjugants

Strain	Date of isolation (mo and yr)	Patient	Source	β-Lactamase	MIC (μg/ml)[a]							Transconjugant (*E. coli* CSH-2)	β-Lactamase	MIC (μg/ml)[a]						
					PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM			PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM
												E. coli CSH-2		1	0.13	0.13	≤0.06	≤0.06	0.5	0.25
K. pneumoniae																				
HKY209	Jul. 95	A	Sputum	CMY-9	32	>128	1	>128	0.25	>128	0.25	*E. coli*(pK209)	CMY-9	4	64	0.25	128	≤0.06	128	0.25
HKY327	Apr. 95	B	Sputum	CMY-19	128	>128	32	128	4	64	0.25	*E. coli*(pK327)	CMY-19	64	>128	8	128	4	32	0.5
HKY363	Jun. 96	C	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK363)	CMY-19	32	>128	8	64	2	16	0.25
HKY466	Oct. 96	D	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK466)	CMY-19	32	>128	8	64	2	16	0.25
HKY474	Jan. 97	E	Sputum	CMY-19	64	>128	16	64	4	64	0.13	*E. coli*(pK474)	CMY-19	32	>128	4	64	4	16	0.25
E. coli																				
HKY154	Mar. 95	F	Sputum	CMY-9 and TEM-1-like[c]	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE154)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY191	Jun. 95	G	Pus	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE191)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY200	Jun. 95	H	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE200)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY215	Jul. 95	H	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE215)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY224	Aug. 95	I	Stool	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE224)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKYM68	Nov. 95	J	Sputum	CMY-9 and TEM-1-like	32	>128	2	>128	2	>128	32[d]	*E. coli*(pEM68)	CMY-9	8	64	0.5	128	≤0.06	128	0.25
HKY297	Mar. 96	K	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.25	>128	0.13	*E. coli*(pE297)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY315	Apr. 96	L	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE315)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY334	Apr. 96	K	Sputum	CMY-9 and TEM-1-like	64	>128	1	>128	0.5	>128	0.13	*E. coli*(pE334)	CMY-9	4	64	0.5	128	≤0.06	128	0.25

[a] Abbreviations: PIP, piperacillin; CAZ, ceftazidime; APB, 3-aminophenyl-boronic acid; CTX, cefotaxime; FEP, cefepime; CMZ, cefmetazole; IPM, imipenem.
[b] APB was used at a concentration of 300 μg/ml.
[c] The nucleotide sequence of the PCR amplicon derived from the *bla* gene was identical to that of the bla_{TEM-1} gene, although the total nucleotide sequence of the *bla* gene was not determined.
[d] Imipenem resistance may be due to alteration in bacterial membrane as reported previously (3, 9, 29).

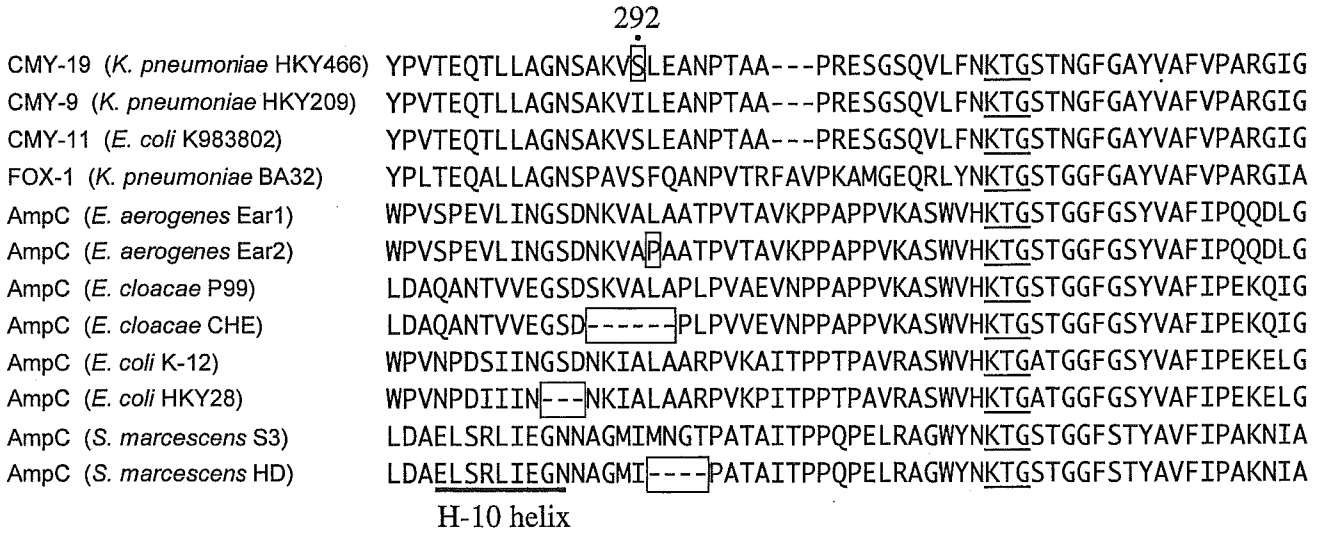

FIG. 1. Alignments of amino acid residues near the H-10 helix. A partial amino acid sequence alignment of CMY-9 (12), CMY-19 (this study), CMY-11 (21), FOX-1 (15), AmpC of *E. cloacae* Ear1 and Ear2 (5), AmpC of *E. cloacae* P99 and HD (6), AmpC of *E. coli* K-12 and HKY28 (13), and AmpC of *S. marcescens* S3 and HD (22) is shown. Square boxes show the amino acid substitutions or deletions that are predicted to affect the hydrolyzing activity of cefepime. The conserved motif KTG is underlined. Dashes indicate deletions of amino acid residues. CMY-11- and FOX-type enzymes have a serine residue at amino acid position 292, but no observation about their property against cefepime was described in the articles. The numbering of the amino acid residues is in reference to that of the mature CMY-1 reported by Bauernfeind et al. (7).

The mechanism of imipenem resistance of HKYM68 was not characterized in this work.

Transferability of β-lactam resistance. The oximino-cephalosporin and cephamycin resistance trait of the five *K. pneumoniae* was transferred to a recipient *E. coli* strain (strain CSH-2) at a frequency of 10^{-4} to 10^{-5} cells per recipient cell by broth mating. Conjugal transfer of the resistance trait from the nine *E. coli* isolates was also observed at a frequency of about 10^{-3} to 10^{-4} cells per recipient cell.

PCR analyses confirmed the presence of bla_{CMY-9} or bla_{CMY-19} in each transconjugant, indicating that these genes are located on transferable plasmids. PCR analysis of the transconjugants also revealed no cotransmission of the bla_{TEM} gene to the *E. coli* transconjugants that harbored the bla_{CMY-9} gene.

PFGE analysis. The PFGE patterns of the five *K. pneumoniae* isolates after XbaI digestion were highly variable (Fig. 2A), which

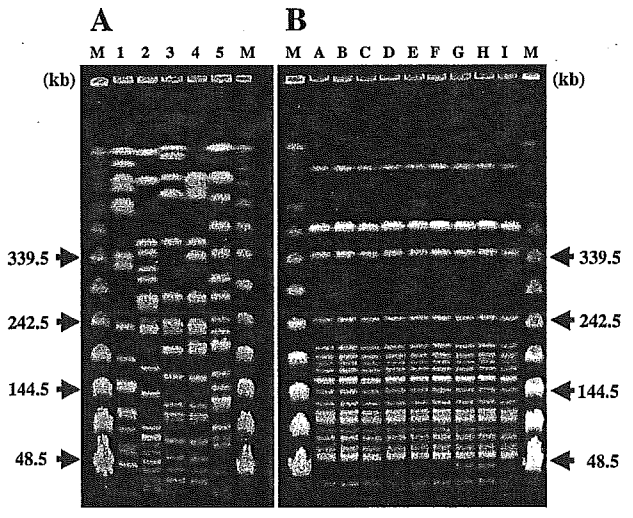

FIG. 2. PFGE analysis of *K. pneumoniae* and *E. coli* isolates. (A) Lanes: M, PFGE marker; 1, *K. pneumoniae* HKY209; 2, *K. pneumoniae* HKY327; 3, *K. pneumoniae* HKY363; 4, *K. pneumoniae* HKY466; 5, *K. pneumoniae* HKY474. (B) Lanes: M, PFGE marker; A, *E. coli* HKY154; B, *E. coli* HKY191; C, *E. coli* HKY200; D, *E. coli* HKY215; E, *E. coli* HKY224; F, *E. coli* HKY297; G, *E. coli* HKY315; H, *E. coli* HKY334; and I, *E. coli* HKYM68.

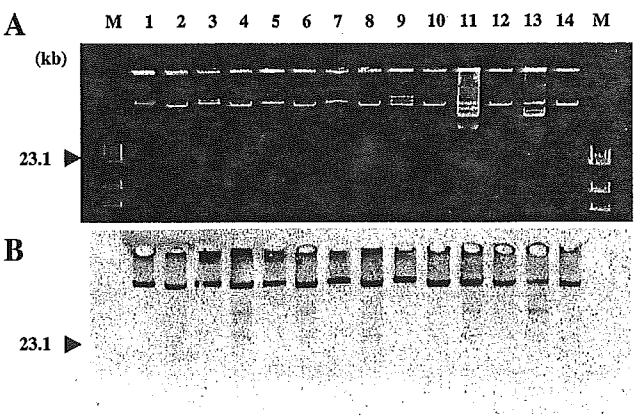

FIG. 3. Plasmid profiles and Southern hybridization. (A) Plasmid profiles of clinical isolates and their tranconjugants; (B) hybridization with the probe specific for the CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *K. pneumoniae* HKY209; 2, *E. coli* CSH-2/pK209; 3, *K. pneumoniae* HKY327; 4, *E. coli* CSH-2/pK327; 5, *K. pneumoniae* HKY363; 6, *E. coli* CSH-2/pK363; 7, *K. pneumoniae* HKY466; 8, *E. coli* CSH-2/pK466; 9, *K. pneumoniae* HKY474; 10, *E. coli* CSH-2/pK474; 11, *E. coli* HKY154; 12, *E. coli* CSH-2/pE154; 13, *E. coli* HKYM68; and 14, *E. coli* CSH-2/pEM68.

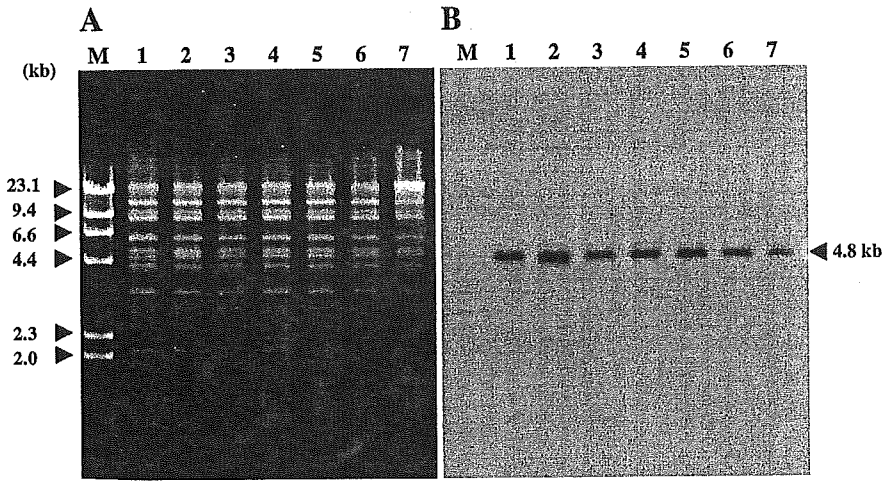

FIG. 4. Plasmid patterns after restriction enzyme digestion and Southern hybridization. (A) SacI-digested plasmid DNAs prepared from the representative transconjugants; (B) hybridization patterns with the probe specific for CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *E. coli* CSH-2/pK209; 2, *E. coli* CSH-2/pK327; 3, *E. coli* CSH-2/pK363; 4, *E. coli* CSH-2/pK466; 5, *E. coli* CSH-2/pK474; 6, *E. coli* CSH-2/pE154; and 7, *E. coli* CSH-2/pEM68.

revealed their clonal diversity. In contrast, the PFGE patterns of the *E. coli* isolates were very similar to one another (Fig. 2B), which revealed their clonal relatedness.

Plasmid analyses and Southern hybridization. The plasmid DNA profiles prepared from the five *K. pneumoniae* isolates and their transconjugants and from two representative *E. coli* isolates (isolates HKY154 and HKYM68) and their transconjugants are shown in Fig. 3A. One to three large plasmids were apparently present in the five *K. pneumoniae* isolates, and one large plasmid was transferred to *E. coli* from each strain in the conjugation experiment. *E. coli* isolate HKY154 had four plasmids, and identical plasmid profiles were found in seven other *E. coli* isolates (isolates HKY191, HKY200, HKY215, HKY224, HKY297, HKY315, and HKY334) (data not shown). *E. coli* HKYM68 harbored three plasmids that were similar to three of the four plasmids found in HKY154. All the *E. coli* transconjugants carried a single plasmid apparently identical to that transferred from the *K. pneumoniae* isolates (Fig. 3A and data not shown).

A plasmid of similar size hybridized with the DNA probe specific for the CMY-1-type β-lactamase genes in all the *K. pneumoniae* and *E. coli* isolates and their transconjugants (Fig. 3B). The SacI restriction profiles of the plasmid DNAs from representative transconjugants were very similar to each other (Fig. 4A), and the DNA probe specific for bla_{CMY-1}-group genes hybridized with a band of about 4.8 kb in size in all cases (Fig. 4B).

Isoelectric focusing of β-lactamases. A β-lactamase band with a pI of >8.45 was detected in all *K. pneumoniae* and *E. coli* clinical isolates and their transconjugants but not in *E. coli* CSH-2 (data not shown). This band likely corresponded to the CMY-9 or CMY-19 β-lactamase. No band with an acidic pI value was detected in the nine *E. coli* clinical isolates carrying the bla_{TEM} gene, likely because of a low level of expression of that gene.

Genetic environments of bla_{CMY-9} and bla_{CMY-19} genes. The structure of the flanking regions of the bla_{CMY-9} gene in *E. coli* HKYM68 was already reported in a previous study (12). The structure surrounding the bla_{CMY} genes in the other 13 isolates was identical to that found in HKYM68. Both bla_{CMY-9} and bla_{CMY-19} were located at the 3′ end of a putative transposase gene, *orf513*. A *sul1*-type class 1 integron structure consisting of *intI1* (an integrase gene), a fused *aacA1-orfG* gene cassette (responsible for aminoglycoside resistance), *qacEΔ1*, and *sul1* (responsible for trimethoprim-sulfamethoxazole resistance) were found at the 5′ end of *orf513* (Fig. 5).

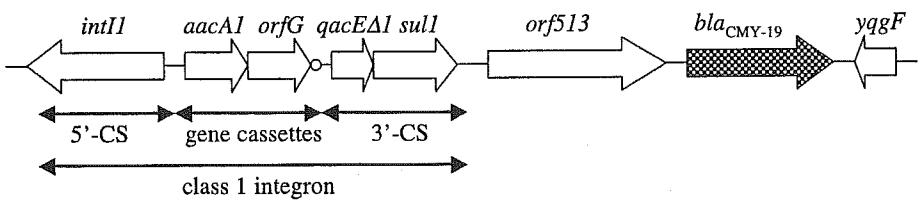

FIG. 5. Gene organization around bla_{CMY} genes. The bla_{CMY} gene on the conjugative plasmid found in the *K. pneumoniae* and *E. coli* clinical isolates located just downstream of *orf513* is shown as it was found in our previous study on a CMY-9 producing *E. coli* HKHM68 (12). Open circle, position of the 59-base element; CS, conserved segment of a class 1 integron. *orf513* is speculated to encode a putative transposase, and various antimicrobial resistance genes tend to be integrated just downstream the *orf513*. The product from the *yqgF* gene encodes a hypothetical protein very similar to the YqgF identified in *Aeromonas hydrophila* (EMBL accession no. AJ276030), but the function is unknown.

TABLE 2. MICs of β-lactams for CMY-9-producing and CMY-19-producing *E. coli* transformants

β-Lactam	MIC (μg/ml)		
	Transformant		Recipient, *E. coli* DH5α (pBCSK+)
	E. coli DH5α (pBC-CMY-9) CMY-9	*E. coli* DH5α (pBC-CMY-19) CMY-19	
Ampicillin	64	>128	2
Piperacillin	8	64	0.5
Piperacillin + TAZ[a]	4	32	0.5
Cephalothin	>128	>128	2
Cephaloridine	64	128	2
Ceftizoxime	64	16	≤0.06
Ceftazidime	64	>128	≤0.06
Ceftazidime + APB[b]	0.5	8	≤0.06
Cefotaxime	>128	128	≤0.06
Cefotaxime + APB[b]	2	1	≤0.06
Cefpirome	8	16	≤0.06
Cefepime	0.13	4	≤0.06
Cefoxitin	>128	128	2
Cefmetazole	128	32	0.5
Cefminox	128	32	0.5
Moxalactam	8	8	≤0.06
Aztreonam	4	16	≤0.06
Imipenem	0.25	0.25	0.13
Meropenem	≤0.06	≤0.06	≤0.06

[a] TAZ, tazobactam, which was used at a concentration of 4 μg/ml.
[b] APB, 3-Aminophenyl boronic acid, which was used at a concentration of 300 μg/ml.

MICs for CMY-9- or CMY-19-producing *E. coli* transformants. The MICs of various β-lactams for CMY-9- or CMY-19-producing *E. coli* transformants are shown in Table 2. Some notable differences were observed between the MICs of the two strains. The MICs of ampicillin and piperacillin for the CMY-19 producer were higher than those for the CMY-9 producer. Concerning ceftizoxime and cefotaxime, the MICs for the CMY-9 producer were higher than those for the CMY-19 producer, but in the case of ceftazidime, the level of resistance was reversed. The CMY-19 producer showed higher levels of resistance to cefpirome and cefepime than the CMY-9 producer. The MICs of cephamycins, such as cefoxitin, cefmetazole, and cefminox, were higher for the CMY-9 producer than for the CMY-19 producer. A remarkable reduction in the MICs by the addition of a class C β-lactamase specific inhibitor, 3-aminophenyl boronic acid, was observed with both the CMY-9 and the CMY-19 producers.

Kinetic parameters. To purify the CMY-9 and the CMY-19 β-lactamases, initially, *E. coli* DH5α(pBC-CMY-9) and *E. coli* DH5α(pBC-CMY-19) were cultured in 2 liters of LB broth. However, the yield of purified CMY-19 β-lactamase was insufficient for the assay of kinetic parameters. Therefore, a pET29a(+) expression vector and an *E. coli* BL21(DE3) pLysS strain were used for overproduction and purification of that enzyme. The purified enzymes gave a single band on SDS-PAGE with CBB staining that suggested >95% purity (data not shown).

The kinetic parameters of CMY-9 and CMY-19 against selected β-lactams are shown in Table 3. The hydrolyzing activity (k_{cat}/K_m) of CMY-19 for penicillins, including ampicillin and piperacillin, were higher than those of CMY-9. Although CMY-9 and CMY-19 had similar k_{cat} values for cefotaxime, CMY-19 had a 100-fold-higher K_m than CMY-9, resulting in a lower catalytic efficiency for this substrate. Ceftazidime, cefpirome, and cefepime behaved as poor substrates for CMY-9 due to the high K_m values for these agents, while CMY-19 showed different behaviors against these compounds. CMY-19 had a 140-fold-lower K_m against ceftazidime than CMY-9. The k_{cat} value of CMY-9 for cefepime could not be determined, but CMY-19 measurably hydrolyzed this compound. The hydrolyzing efficiencies (k_{cat}/K_m values) of CMY-19 against cephamycins such as cefoxitin and cefmetazole were lower than those of CMY-9. Although CMY-19 had a lower K_m against cephamycins than CMY-9, it showed a much lower k_{cat} against these compounds.

DISCUSSION

A plasmid-mediated class C β-lactamase (CMY-1) was first reported in 1989 in a *K. pneumoniae* isolated in South Korea (8). Subsequently, several variants of that enzyme, such as MOX-1 (16), CMY-8 (33), CMY-9 (12), CMY-10 (20), and CMY-11 (21), have been identified, mainly in East Asian countries, including Taiwan and Japan. The dissemination of CMY-

TABLE 3. Kinetic parameters of CMY-9 and CMY-19

Substrate	CMY-9			CMY-19		
	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Ampicillin	91 ± 28	1.0 ± 0.1	1.1×10^4	16 ± 1	0.35 ± 0.01	2.2×10^4
Piperacillin	97 ± 21	0.14 ± 0.01	1.4×10^3	8.9 ± 0.5	0.031 ± 0.001	3.5×10^3
Cephalothin	120 ± 10	630 ± 10	5.3×10^6	230 ± 10	380 ± 10	1.7×10^6
Cephaloridine	1200 ± 100	99 ± 2	8.3×10^4	1500 ± 100	240 ± 10	1.6×10^5
Ceftizoxime	5.5 ± 0.2	1.3 ± 0.1	2.4×10^5	11 ± 1	0.71 ± 0.03	6.5×10^4
Ceftazidime	560 ± 110	1.8 ± 0.3	3.2×10^3	3.7 ± 0.1	0.085 ± 0.002	2.3×10^4
Cefotaxime	0.28 ± 0.01	0.27 ± 0.01	9.6×10^5	31 ± 2	0.33 ± 0.01	1.1×10^4
Cefpirome	390 ± 50	3.6 ± 0.3	9.2×10^3	25 ± 2	0.58 ± 0.02	2.3×10^4
Cefepime	950 ± 50	NH[a]	ND[b]	630 ± 170	1.8 ± 0.4	2.9×10^3
Cefoxitin	60 ± 2	50 ± 1	8.3×10^5	0.90 ± 0.03	0.12 ± 0.01	1.3×10^5
Cefmetazole	5.1 ± 0.2	1.7 ± 0.1	3.3×10^5	0.26 ± 0.01	0.045 ± 0.001	1.7×10^5
Moxalactam	0.22 ± 0.01	NH	ND	0.40 ± 0.03	NH	ND
Imipenem	4.6 ± 0.3	NH	ND	4.3 ± 0.1	NH	ND

[a] NH, not hydrolyzed.
[b] ND, not determined.

10- and CMY-11-producing isolates of the family *Enterobacteriaceae* was also reported in Korea (18, 19). In the present study, CMY-type β-lactamase-producing *K. pneumoniae* and *E. coli* clinical isolates from a Japanese general hospital were investigated. Through PFGE analysis, it was found that the five *K. pneumoniae* isolates had little genetic relatedness to each other, while the nine *E. coli* isolates belonged to the same clonal lineage. Interestingly, plasmid analysis showed that all 14 isolates harbored a very similar conjugal plasmid that encodes a CMY-type β-lactamase which was either CMY-9 or CMY-19, a variant that differs from CMY-9 by a single amino acid substitution (I292S). Since the flanking structures of the bla_{CMY} genes were identical in all plasmids, it is probable that one conjugal plasmid carrying bla_{CMY-9} was horizontally transferred to *E. coli*, *K. pneumoniae*, and then an *E. coli* clone and various *K. pneumoniae* strains harboring the bla_{CMY} gene-bearing plasmids might have spread in the hospital.

β-Lactamases can modify their substrate specificity through a single amino acid substitution (30). CMY-19 had a single amino acid substitution, I292S, near the H-10 helix domain, compared with the sequence of CMY-9 (Fig. 1). Indeed, a serine residue at the same amino acid position was found in all the FOX-type enzymes (15, 26), including CAV-1 (14), and also in CMY-11 (21), as shown Fig. 1; but no peculiar behavior against cefepime was documented with those enzymes. Through the I292S substitution, CMY-19 would have developed extended substrate specificity against cefepime and cefpirome, as well as ampicillin, piperacillin, cephaloridine, and ceftazidime, compared with that of CMY-9, although the hydrolyzing activities against ceftizoxime, cefotaxime, and cephamycins were impaired. The expansion of hydrolyzing activity against cefepime found in CMY-19 was a most remarkable property because cefepime is generally stable against AmpC β-lactamases (11). Similar developments of extended hydrolyzing activity against cefepime through amino acid substitutions or deletions adjacent to the H-10 helix have been observed in several chromosomally encoded AmpC β-lactamases, such as the AmpC of *Serratia marcescens* that lacks four amino acids at positions 293 to 296 (22), an AmpC of *E. coli* that lacks three amino acids at positions 286 to 288 (13), an AmpC of *Enterobacter cloacae* that lacks six amino acids at positions 289 to 294 (6), and an AmpC of *Enteroacter aerogenes* that acquired an L293P substitution (5). Furthermore, Barlow and Hall reported on the in vitro selection of CMY-2 β-lactamase variants with several amino acid substitutions, including replacements at positions 292, 293, 294, 296, and 298, which demonstrated increased resistance to cefepime (4). From our previous molecular modeling analyses (13), it was found that the expansion of an open space in the vicinity of the R-2 side chain of ceftazidime or cefepime through the deletion of tripeptides in the H-10 helix of *E. coli* AmpC (Fig. 1) played a crucial role in the acquisition of the greater hydrolyzing activity against those agents possessing a bulky R side chain. Although the actual mechanism for the higher cefepime-hydrolyzing activity in CMY-19 compared to that in CMY-9 has not been elucidated, a similar molecular distortion at the active center of the enzyme might well have occurred in CMY-19. This speculation would be substantiated by molecular modeling and X-ray crystallographic analyses.

ACKNOWLEDGMENTS

We are grateful to Kumiko Kai and Fusako Yokokawa for technical assistance.

This work was supported by the Ministry of Health, Labor and Welfare of Japan (grants H15-Shinkou-9 and H15-Shinkou-10). The research activity of J. Wachino was supported by a Scholarship for Young Scientists provided by the Japan Society for the Promotion of Science.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. London B Biol. Sci. **289:**321–331.
2. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38:**40–43.
3. **Armand-Lefevre, L., V. Leflon-Guibout, J. Bredin, F. Barguellil, A. Amor, J. M. Pages, and M. H. Nicolas-Chanoine.** 2003. Imipenem resistance in *Salmonella enterica* serovar Wien related to porin loss and CMY-4 β-lactamase production. Antimicrob. Agents Chemother. **47:**1165–1168.
4. **Barlow, M., and B. G. Hall.** 2003. Experimental prediction of the evolution of cefepime resistance from the CMY-2 AmpC β-lactamase. Genetics **164:** 23–29.
5. **Barnaud, G., Y. Benzerara, J. Gravisse, L. Raskine, M. J. Sanson-Le Pors, R. Labia, and G. Arlet.** 2004. Selection during cefepime treatment of a new cephalosporinase variant with extended-spectrum resistance to cefepime in an *Enterobacter aerogenes* clinical isolate. Antimicrob. Agents Chemother. **48:**1040–1042.
6. **Barnaud, G., R. Labia, L. Raskine, M. J. Sanson-Le Pors, A. Philippon, and G. Arlet.** 2001. Extension of resistance to cefepime and cefpirome associated to a six amino acid deletion in the H-10 helix of the cephalosporinase of an *Enterobacter cloacae* clinical isolate. FEMS Microbiol. Lett. **195:**185–190.
7. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, R. Wilhelm, and Y. Chong.** 1996. Comparative characterization of the cephamycinase bla_{CMY-1} gene and its relationship with other β-lactamase genes. Antimicrob. Agents Chemother. **40:**1926–1930.
8. **Bauernfeind, A., Y. Chong, and S. Schweighart.** 1989. Extended broad-spectrum β-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection **17:**316–321.
9. **Bradford, P. A., C. Urban, N. Mariano, S. J. Projan, J. J. Rahal, and K. Bush.** 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob. Agents Chemother. **41:**563–569.
10. **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.
11. **Chapman, T. M., and C. M. Perry.** 2003. Cefepime: a review of its use in the management of hospitalized patients with pneumonia. Am. J. Respir. Med. **2:**75–107.
12. **Doi, Y., N. Shibata, K. Shibayama, K. Kamachi, H. Kurokawa, K. Yokoyama, T. Yagi, and Y. Arakawa.** 2002. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **46:**2427–2434.
13. **Doi, Y., J. Wachino, M. Ishiguro, H. Kurokawa, K. Yamane, N. Shibata, K. Shibayama, K. Yokoyama, H. Kato, T. Yagi, and Y. Arakawa.** 2004. Inhibitor-sensitive AmpC β-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob. Agents Chemother. **48:**2652–2658.
14. **Fosse, T., C. Giraud-Morin, I. Madinier, and R. Labia.** 2003. Sequence analysis and biochemical characterisation of chromosomal CAV-1 (*Aeromonas caviae*), the parental cephalosporinase of plasmid-mediated AmpC 'FOX' cluster. FEMS Microbiol. Lett. **222:**93–98.
15. **Gonzalez Leiza, M., J. C. Perez-Diaz, J. Ayala, J. M. Casellas, J. Martinez-Beltran, K. Bush, and F. Baquero.** 1994. Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated β-lactamase with two molecular variants. Antimicrob. Agents Chemother. **38:**2150–2157.
16. **Horii, T., Y. Arakawa, M. Ohta, S. Ichiyama, R. Wacharotayankun, and N. Kato.** 1993. Plasmid-mediated AmpC-type β-lactamase isolated from *Klebsiella pneumoniae* confers resistance to broad-spectrum β-lactams, including moxalactam. Antimicrob. Agents Chemother. **37:**984–990.
17. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
18. **Kim, J. Y., J. S. Song, S. H. Bak, Y. E. Cho, D. W. Kim, S. H. Jeong, Y. M. Park, K. J. Lee, and S. H. Lee.** 2004. Dissemination of *Escherichia coli* producing AmpC-type β-lactamase (CMY-11) in Korea. Int. J. Antimicrob. Agents **24:**320–326.

19. **Lee, J. H., H. I. Jung, J. H. Jung, J. S. Park, J. B. Ahn, S. H. Jeong, B. C. Jeong, and S. H. Lee.** 2004. Dissemination of transferable AmpC-type β-lactamase (CMY-10) in a Korean hospital. Microb. Drug Resist. **10:**224–230.
20. **Lee, S. H., S. H. Jeong, and Y. M. Park.** 2003. Characterization of bla_{CMY-10}, a novel plasmid-encoded AmpC-type β-lactamase gene in a clinical isolate of *Enterobacter aerogenes*. J. Appl. Microbiol. **95:**744–752.
21. **Lee, S. H., J. Y. Kim, G. S. Lee, S. H. Cheon, Y. J. An, S. H. Jeong, and K. J. Lee.** 2002. Characterization of bla_{CMY-11}, an AmpC-type plasmid-mediated β-lactamase gene in a Korean clinical isolate of *Escherichia coli*. J. Antimicrob. Chemother. **49:**269–273.
22. **Mammeri, H., L. Poirel, P. Bemer, H. Drugeon, and P. Nordmann.** 2004. 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. Antimicrob. Agents Chemother. **48:**716–720.
23. **Nakano, R., R. Okamoto, Y. Nakano, K. Kaneko, N. Okitsu, Y. Hosaka, and M. Inoue.** 2004. CFE-1, a novel plasmid-encoded AmpC β-lactamase with an *ampR* gene originating from *Citrobacter freundii*. Antimicrob. Agents Chemother. **48:**1151–1158.
24. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Document M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
25. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
26. **Queenan, A. M., S. Jenkins, and K. Bush.** 2001. Cloning and biochemical characterization of FOX-5, an AmpC-type plasmid-encoded β-lactamase from a New York City *Klebsiella pneumoniae* clinical isolate. Antimicrob. Agents Chemother. **45:**3189–3194.
27. **Sanders, C. C.** 1987. Chromosomal cephalosporinases responsible for multiple resistance to newer β-lactam antibiotics. Annu. Rev. Microbiol. **41:**573–593.
28. **Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa.** 2003. PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol. **41:** 5407–5413.
29. **Stapleton, P. D., K. P. Shannon, and G. L. French.** 1999. Carbapenem resistance in *Escherichia coli* associated with plasmid-determined CMY-4 β-lactamase production and loss of an outer membrane protein. Antimicrob. Agents Chemother. **43:**1206–1210.
30. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, and Y. Arakawa.** 2004. Molecular characterization of a cephamycin-hydrolyzing and inhibitor-resistant class A β-lactamase, GES-4, possessing a single G170S substitution in the omega-loop. Antimicrob. Agents Chemother. **48:**2905–2910.
31. **Wachino, J., Y. Doi, K. Yamane, N. Shibata, T. Yagi, T. Kubota, H. Ito, and Y. Arakawa.** 2004. Nosocomial spread of ceftazidime-resistant *Klebsiella pneumoniae* strains producing a novel class a β-lactamase, GES-3, in a neonatal intensive care unit in Japan. Antimicrob. Agents Chemother. **48:** 1960–1967.
32. **Yagi, T., J. Wachino, H. Kurokawa, K. Yamane, Y. Doi, N. Shibata, H. Kato, K. Shibayama, and Y. Arakawa.** 2004. Practical methods for identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* using boronic acid compounds. J. Clin. Microbiol. **43:**2551–2558.
33. **Yan, J. J., S. M. Wu, S. H. Tsai, J. J. Wu, and I. J. Su.** 2000. Prevalence of SHV-12 among clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases and identification of a novel AmpC enzyme (CMY-8) in southern Taiwan. Antimicrob. Agents Chemother. **44:**1438–1442.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2006, p. 791–795
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.2.791–795.2006

Vol. 50, No. 2

PCR Classification of CTX-M-Type β-Lactamase Genes Identified in Clinically Isolated Gram-Negative Bacilli in Japan

Naohiro Shibata, Hiroshi Kurokawa, Yohei Doi, Tetsuya Yagi, Kunikazu Yamane, Jun-ichi Wachino, Satowa Suzuki, Kouji Kimura, Satoshi Ishikawa, Haru Kato, Yoshiyuki Ozawa, Keigo Shibayama, Kumiko Kai, Toshifumi Konda, and Yoshichika Arakawa*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan

Received 26 March 2005/Returned for modification 15 June 2005/Accepted 1 November 2005

Of 1,456 strains isolated from 2001 to 2003 demonstrating resistance to either oxyimino-cephalosporin, 317 strains, isolated in 57 of 132 clinical facilities, were found to harbor bla_{CTX-M} genes by PCR. Fifty-seven, 161, and 99 strains harbored bla_{CTX-M} genes belonging to the $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ clusters, respectively.

In recent years, CTX-M-type β-lactamases have been recognized as a growing family possessing a high level of hydrolyzing activities, especially against cefotaxime (CTX) and ceftriaxone. Nearly 40 variants of the CTX-M-type enzymes have been identified (2, 4, 13, 25) and registered to date (http://www.lahey.org/studies/other.asp#table_1). Further proliferation of CTX-M-type β-lactamase-producing gram-negative bacteria has become a great concern (6), since a large number of nosocomial outbreaks caused by such bacteria have so far been recognized and reported in various medical facilities in many countries (1, 3, 5, 7–9, 19, 21).

In Japan, FEC-1 and Toho-1 were initially identified (12, 15) and were later included in CTX-M-type enzymes. Since then, various strains that produce a Toho-1-like β-lactamase have been identified in Japanese clinical settings (26, 28). Almost all of them, however, were found to be CTX-M-2 by sequence analyses (N. Shibata, et al. Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2235, 2001). However, the trends for several CTX-M-type β-lactamases other than CTX-M-2 have remained unclear. In the present study, we investigated the molecular types of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated in Japanese clinical facilities using PCR methods.

From January 2001 to December 2003, 1,456 gram-negative bacterial isolates demonstrating resistance to oxyimino-cephalosporins were submitted from 132 hospitals to the reference laboratory at our institute. These strains were then subjected to screening for β-lactamases, including TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs), CTX-M-type β-lactamases, AmpC- and CMY-type class C cephalosporinases and cephamycinases, and class B metallo-β-lactamases (MBLs). The strains were checked for ESBL production by the double-disk diffusion synergy test recommended by the CLSI (formerly the NCCLS) (18). The MICs of ceftazidime (CAZ) and CTX for the clinical isolates were determined by the agar dilution method recommended by the CLSI guidelines. When a clinical isolate demonstrated resistance to either oxyimino-cephalosporin, the strain was then subjected to PCR analyses for detection of bla_{CTX-M} genes. PCR analysis was performed by the method reported previously (27). The four sets of PCR primers used for detection of bla_{CTX-M} genes in the present study were as follows: primers CTX-M-1-F (5′-GCT GTT GTT AGG AAG TGT GC-3′) and CTX-M-1-R (5′-CCA TTG CCC GAG GTG AAG-3′), primers CTX-M-2-F (5′-ACG CTA CCC CTG CTA TTT-3′) and CTX-M-2-R (5′-CCT TTC CGC CTT CTG CTC-3′), primers CTX-M-8-F (5′-CGG ATG ATG CTA ATG ACA AC-3′) and CTX-M-8-R (5′-GTC AGA TTG CGA AGC GTC-3′), and primers CTX-M-9-F (5′-GCA GAT AAT ACG CAG GTG-3′) and CTX-M-9-R (5′-CGG CGT GGT GGT GTC TCT-3′). Only one strain was selected from an individual patient and subjected to the PCR test.

As shown in Table 1, the inhibition patterns by combination of the double-disk diffusion synergy test for ESBL detection and the sodium mercaptoacetic acid (SMA) disk test for MBL detection were classified into four groups. Of 1,456 strains tested, 59 were resistant only to CAZ and susceptible to clavulanic acid. It was speculated that these strains produce mainly SHV- or TEM-derived ESBLs, because SHV-12-producing strains have been prevalent in Japan (27). On the other hand, 276 strains showed resistance to CTX but were susceptible to CAZ. The MIC of CTX was significantly decreased in the presence of clavulanic acid. It was speculated that these strains chiefly produce CTX-M-type β-lactamases. Five hundred forty-eight isolates demonstrated resistance to both CAZ and CTX; but the inhibitory effect of clavulanic acid was not clear in these strains, and the production of MBL was suggested, because the MICs of CAZ and CTX were reduced in the presence of SMA, which is a specific inhibitor of metallo-β-lactamase (23). The remaining 573 strains, which demonstrated resistance to either of the oxyimino-cephalosporins, did not become susceptible to these agents in the presence of SMA, suggesting the production of some AmpC-type enzymes, including plasmid-mediated CMY-type enzymes.

Of 1,397 strains subjected to the PCR analyses, 317 strains were suggested to harbor bla_{CTX-M} genes. Of these strains, 57 appeared to carry genes of the $bla_{CTX-M-1}$ group, including

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

TABLE 1. Results of screening by double-disk diffusion synergy tests

Bacterial species	Pattern of double-disk diffusion synergy test				Total no. of strains tested
	Resistant to CAZ and susceptible to clavulanic acid (no. of strains)	Resistant to CTX and susceptible to clavulanic acid[a]	Resistant to CAZ and CTX and susceptible to SMA[a]	Resistant to either oxyimino-cephalosporin and not susceptible to SMA	
Escherichia coli	33	157/157	7/24	4/4	218
Proteus mirabilis	0	71/71	0/1	0/0	72
Klebsiella pneumoniae	15	42/42	7/31	1/2	90
Klebsiella oxytoca	4	5/5	1/3	0/2	14
Serratia marcescens	7	0/0	0/65	10/77	149
Enterobacter cloacae	0	0/0	2/11	1/20	31
Enterobacter aerogenes	0	0/0	0/2	1/8	10
Citrobacter freundii	0	0/0	0/4	2/15	19
Citrobacter koseri	0	0/0	0/0	1/1	1
Providencia rettgeri	0	1/1	0/2	0/0	3
Acinetobacter baumannii	0	0/0	1/49	3/40	89
Other bacterial species[d]	0	0/0	0/356	0/404	760
Total[e]	59	276/276	18/548[b]	23/573[c]	1,456

[a] The data represent the number of bla_{CTX-M}-positive strains by PCR/total number of strains demonstrating each inhibition pattern and subjected to PCR.
[b] Strains that produce metallo-β-lactamase are included.
[c] Strains that produce plasmid-mediated CMY-type cephalosporinase or chromosomal AmpC hyperproducers are included.
[d] *Pseudomonas* spp., *Alcaligenes* spp., *Achromobacter* spp., and *Burkholderia* spp. demonstrating resistance to ceftazidime or cefotaxime were included; but *Stenotrophomonas* spp. and *Chryseobacterium* spp. that produce intrinsic metallo-β-lactamase were excluded.
[e] Out of the total number of strains being subjected to PCR analysis (1,397; represented in columns 2, 3, and 4), 317 were found to be bla_{CTX-M} positive.

$bla_{CTX-M-1}$, $bla_{CTX-M-3}$, and $bla_{CTX-M-15}$, as shown in Table 2. Moreover, 161 strains were suggested to harbor the genes encoding the CTX-M-2 group of enzymes, such as CTX-M-2, CTX-M-20, and CTX-M-31. Furthermore, 99 strains appeared to carry the genes for the CTX-M-9 group of enzymes, such as CTX-M-9, CTX-M-14, and CTX-M-16. No strain harboring genes for the CTX-M-8 or the CTX-M-25 group of enzymes was found among the strains tested.

As shown in Table 3, strains that harbored genes for the CTX-M-type enzymes were isolated from 57 of 132 hospitals across Japan, except for the Hokkaido region, throughout the 3-year investigation period. Fourteen and 24 strains that harbored genes for the CTX-M-1 group of enzymes were identified in 7 and 10 hospitals located in the Kanto and Chubu regions, respectively (Table 3). However, no strain harboring genes for the CTX-M-1 group of enzymes were found in the Chugoku and Shikoku regions (Table 3). In 22 of 57 hospitals, genes for multiple CTX-M-type β-lactamases belonging to different groups were identified during the investigation period (Fig. 1). Interestingly, genes for all three groups of CTX-M-type enzymes were identified in 7 of 57 hospitals (Fig. 1; Table 3).

TABLE 2. Number of strains that produce CTX-M-type β-lactamases as detected by PCR

Bacterial species	No. of strains by the following PCR type:			Total
	CTX-M-1 group[a]	CTX-M-2 group[b]	CTX-M-9 group[c]	
Escherichia coli	33	46	89	168
Proteus mirabilis	0	71	0	71
Klebsiella pneumoniae	10	31	9	50
Klebsiella oxytoca	2	3	1	6
Serratia marcescens	9	1	0	10
Enterobacter cloacae	0	3	0	3
Enterobacter aerogenes	1	0	0	1
Citrobacter freundii	2	0	0	2
Citrobacter koseri	0	1	0	1
Providencia rettgeri	0	1	0	1
Acinetobacter baumannii	0	4	0	4
Total	57	161	99	317

[a] The PCR primers used can detect genes for CTX-M-1 and several variants, such as CTX-M-3 and CTX-M-15.
[b] The PCR primers used can detect genes for CTX-M-2 and several variants, such as CTX-M-20 and CTX-M-31.
[c] The PCR primers used can detect genes for CTX-M-9 and several variants, such as CTX-M-14 and CTX-M-16.

After the first description of Toho-1 in Japan in 1995, several outbreaks caused by CTX-M-type β-lactamase producers have been reported in there (17, 26, 28). In the present investigation, it became clear that gram-negative nosocomial bacilli producing the CTX-M-1, CTX-M-2, or CTX-M-9 group of enzymes have already been dispersed in various clinical settings in Japan, although strains that produce TEM- or SHV-derived ESBLs are not predominant to date.

Recently, the CTX-M-1 group of enzymes, such as CTX-M-3 and CTX-M-15, have emerged in Europe and Asia (3, 8–10, 14, 22, 28). In the present study, we also identified the genes for the CTX-M-1 group of enzymes in various bacterial species, including *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, in addition to *Providencia rettgeri*, *Citrobacter freundii*, *Citrobacter koseri*, and *Enterobacter cloacae*. This finding may be suggestive of the lateral transfer of very similar plasmids bearing bla_{CTX-M} genes among different bacterial species. Actually, probable nosocomial transmissions of CTX-M-producing bacterial strains were suspected in several medical facilities, as shown in Fig. 1 and Table 3. Especially in hospitals D18, D20, and E5, all three groups of genes for CTX-M enzymes were identified; and genes for CTX-M-type enzymes were detected in various gram-negative bacterial species, suggesting the horizontal transfer of the bla_{CTX-M} genes among different bacterial species. Interestingly,

Region	PCR type	Bacterial species (no. of isolates)	Hospital (no. of isolates)
Hokkaido (0[a]/7[b])		None	None
Tohoku (4/17)	CTX-M-1	*K. pneumoniae* (2[c])	B4 (2[c])
	CTX-M-2	*E. coli* (1)	B1 (1)
		P. mirabilis (10)	B4 (10)
	CTX-M-9	*E. coli* (6)	B2 (1), B3 (4), B4 (1)
Kanto (9/26)	CTX-M-1	*E. coli* (7)	C1 (1), C3 (2), C9 (4)
		K. pneumoniae (6)	C2 (2), C6 (1), C7 (3)
		K. oxytoca (1)	C8 (1)
	CTX-M-2	*P. mirabilis* (28)	C4 (9), C5 (19)
		A. baumannii (3)	C5 (3)
	CTX-M-9	*K. pneumoniae* (1)	C7 (1)
		E. coli (11)	C2 (1), C3 (1), C4 (1), C7 (4), C8 (4)
Chubu (22/37)	CTX-M-1	*E. coli* (12)	D2 (1), D3 (5), D6 (3), D7 (1), D20 (1), D22 (1)
		K. pneumoniae (2)	D1 (1), D20 (1)
		C. freundii (2)	D18 (2)
		E. aerogenes (1)	D19 (1)
		S. marcescens (5)	D18 (5)
	CTX-M-2	*E. coli* (29)	D5 (1), D6 (2), D8 (1), D13 (4), D14 (1), D15 (5), D18 (1), D20 (14)
		K. pneumoniae (21)	D20 (20), D22 (1)
		K. oxytoca (3)	D6 (1), D15 (1), D20 (1)
		P. mirabilis (17)	D14 (4), D16 (11), D17 (1), D18 (1)
		S. marcescens (1)	D20 (1)
		E. cloacae (3)	D18 (1), D20 (2)
		A. baumannii (1)	D20 (1)
	CTX-M-9	*E. coli* (34)	D4 (1), D5 (1), D6 (4), D7 (4), D8 (4), D9 (3), D10 (1), D11 (1), D12 (1), D14 (1), D16 (4), D18 (1), D20 (3), D21 (5)
		K. pneumoniae (4)	D12 (4)
		K. oxytoca (1)	D12 (1)
Kinki (10/19)	CTX-M-1	*E. coli* (6)	E5 (4), E7 (1), E10 (1)
		K. oxytoca (1)	E4 (1)
		S. marcescens (4)	E1 (4)
	CTX-M-2	*E. coli* (8)	E3 (1), E5 (6), E8 (1)
		K. pneumoniae (6)	E5 (6)
		P. mirabilis (15)	E2 (1), E5 (14)
		P. rettgeri (1)	E8 (1)
	CTX-M-9	*E. coli* (11)	E2 (2), E3 (1), E5 (6), E6 (1), E9 (1)
		K. pneumoniae (2)	E2 (1), E5 (1)
Chugoku (5/13)	CTX-M-2	*E. coli* (3)	F2 (2), F5 (1)
		K. pneumoniae (2)	F3 (2)
	CTX-M-9	*E. coli* (8)	F1 (4), F4 (1), F5 (3)
Shikoku (3/5)	CTX-M-2	*E. coli* (1)	G2 (1)
		C. koseri (1)	G3 (1)
	CTX-M-9	*E. coli* (15)	G2 (15)
		K. pneumoniae (2)	G1 (1), G2 (1)
Kyushu and Okinawa (4/8)	CTX-M-1	*E. coli* (8)	H1 (1), H2 (6), H3 (1)
	CTX-M-2	*E. coli* (4)	H1 (1), H4 (3)
		K. pneumoniae (2)	H4 (2)
		P. mirabilis (1)	H2 (1)
	CTX-M-9	*E. coli* (4)	H1 (3), H2 (1)
Total (57/132)			

[a] Number of medical facilities where bla_{CTX-M}-harboring strains were detected.
[b] Number of medical facilities that submitted strains to our laboratory.
[c] Number of clinical isolates harboring bla_{CTX-M} gene.