TABLE 5. Susceptibility of MBL-producing isolates to various antimicrobial agents

Antimicrobial agent[a]	MIC (μg/ml) for organism								
	Enterobacteriaceae (*n* = 53)			*P. aeruginosa* (*n* = 22)			*Acinetobacter* spp. (*n* = 21)		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Ceftazidime	16–>128	>128	>128	64–>128	128	>128	128–>128	>128	>128
Piperacillin	8–>128	16	>128	4–>128	16	>128	8–>128	32	128
Piperacillin-tazobactam[a]	≤1–>128	8	16	2–>128	8	>128	≤1–32	≤1	4
Cefepime	2–>128	64	128	32–>128	64	>128	64–>128	128	128
Cefoperazone-sulbactam	32–>128	>128	>128	64–>128	64	>128	≤1–16	2	4
Aztreonam	≤1–64	16	32	2–>128	8	32	4–32	16	32
Cefmetazole	>128–>128	>128	>128	>128–>128	>128	>128	64–>128	>128	>128
Latamoxef	64–>128	>128	>128	>128–>128	>128	>128	>128–>128	>128	>128
Meropenem	1–>32	>32	>32	16–>32	32	>32	8–>32	32	>32
Imipenem	1–>32	32	>32	1–>32	16	32	8–>32	16	32
Gentamicin	≤1–4	≤1	2	2–>8	>8	>8	≤1–>8	8	>8
Amikacin	≤4–>32	16	32	≤4–>32	16	32	≤4–>32	8	16
Minocycline	≤4–>8	≤4	>4	>8–>8	>8	>8	≤4–≤4	≤4	≤4
Levofloxacin	≤2–>4	≤2	4	≤2–>4	4	>4	≤2–4	≤2	≤2
Sulfamethoxazole-trimethoprim	≤0.5–>2	≤0.5	>2	>2–>2	>2	>2	≤0.5–>2	>2	>2
Chloramphenicol	≤8–>16	16	>16	>16–>16	>16	>16	≤8–>16	>16	>16

[a] Tazobactam was tested at a fixed concentration of 4 μg/ml.

first reported data on the prevalence of bacteria carrying the genes for MBLs, including IMP-1, IMP-2, and VIM-2 MBLs, in the western portion of Japan.

A total of 96 MBL-positive isolates were typed by RAPD analysis to determine the stabilities of the strain genotypes. The RAPD typing results are summarized in Table 4. The 16 isolates of *P. aeruginosa* from hospital B yielded seven different RAPD patterns and originated from six different wards. Eight *A. baumannii* isolates from hospital A had the same RAPD pattern and were from the same ward. The five *A. baumannii* isolates carrying the genes for IMP-2-group MBLs, isolated from two wards of hospital J, appeared to be of the same clonal lineage. This is the first report of nosocomial spread of *A. baumannii* isolates carrying genes for IMP-2-group MBLs in Japan. Of the 41 *S. marcescens* isolates from hospital C, 13 isolates from the internal medicine coronary care unit had the same RAPD pattern and were isolated within a 5-month period, suggesting that there was probable nosocomial spread within the same ward. Thus, the same or closely related isolates were identified repeatedly by PCR fingerprinting by RAPD analysis from five hospitals, suggesting the nosocomial spread of these organisms in each hospital. Furthermore, long-term cross-transmission of plasmids that carry MBL genes among different bacterial strains and species could result in the current complicated features of MBL producers, especially in hospital B.

The 2-MPA test, which is a simple test that was first described by Arakawa et al. (2), is a useful method for the routine laboratory detection of MBLs (45). Moreover, in the present study, all isolates that tested positive in the 2-MPA test were subsequently confirmed to be positive for the MBL gene by PCR. However, the growth inhibition zones of bla_{VIM-2}-positive *E. cloacae* isolates were weak and ambiguous, possibly due to the excessive production of AmpC and/or a change in membrane permeability. The production of some extended-spectrum β-lactamases as well as the excessive production of the chromosomal AmpC cephalosporinase could be responsible for the characteristics of these strains that were previously reported for *E. cloacae* (2, 7). In such cases, imipenem and meropenem disks would be better than ceftazidime disks for the detection of MBL production because imipenem and meropenem are essentially not hydrolyzed by extended-spectrum β-lactamases and class C cephalosporinases.

With respect to antimicrobial susceptibilities, various β-lactam antimicrobial agents such as ureidopenicillin, cephalosporins, cephamycins, and carbapenems had high MICs for most MBL-positive isolates, whereas monobactam and piperacillin typically had low MICs for MBL producers. Low MICs of cefepime, meropenem, and imipenem were observed for several isolates, even though MBLs can hydrolyze these agents. The production of MBLs in these isolates could be cryptic or suppressed in strains showing low-level carbapenem resistance (14). It is also possible that IMP-3 and IMP-6 MBLs, which have low-level hydrolytic activities against these agents (15, 47), are produced in such isolates. The increased ability of active efflux systems and decreased outer membrane permeabilities have been reported to contribute to β-lactam resistance in *P. aeruginosa* (23, 24). Therefore, the low-level MICs of piperacillin, cefepime, and carbapenems for some isolates may be due to higher permeability coefficients or less efficient efflux pumps in the bacterial membranes in addition to the molecular mechanisms described above.

The MICs of monobactam and piperacillin for MBL producers were relatively low compared to those of oximinocephalosporins, cephamycins, and carbapenems (35, 36); however, this finding does not necessarily reflect their clinical efficacy against MBL producers because most gram-negative rods have the intrinsic ability to produce chromosomal AmpC cephalosporinases, which can hydrolyze monobactam and piperacillin (17). Although the administration of high doses of aztreonam or tazobactam-piperacillin was reported to be useful for the reduction of MBL-producing strains in rats suffering from experimental pneumonia (3), it is possible that the induction of intrinsic chromosomal AmpC production in MBL producers may promote the emergence of multiple-β-lactam-resistant gram-negative rods in clinical settings.

In the present study, MICs of tazobactam-piperacillin and cefoperazone-sulbactam were generally low for MBL-positive *Acinetobacter* isolates. Strains producing IMP-1 or VIM-2 usually show high-level resistance to oximinocephalosporins and cephamycins, but the MIC of piperacillin for these strains is usually lower than those of oximinocephalosporins and cephamycins (9, 30, 32). Because the activities of MBLs are not reduced significantly by β-lactamase inhibitors, such as sulbactam and tazobactam (5), the observations for *Acinetobacter* isolates suggested that the phenotypes related to these combination drugs may depend mainly on the intrinsic production of AmpC cephalosporinase (4, 10) as well as the low-level production of MBLs and alterations in membrane permeability. Thus, the low MIC levels of tazobactam-piperacillin and cefoperazone-sulbactam for MBL-producing *Acinetobacter* isolates could be an intrinsic feature of this bacterial genus.

In conclusion, plasmid-mediated MBL-producing gram-negative rods were first described approximately 13 years ago in Japan, and in the present study, such isolates were found to have disseminated to many hospitals in the Kinki region of Japan. It is conceivable that several isolates have spread nosocomially among a number of hospitals. The results of the present study should be considered when health care facilities develop policies and strategic practices to prevent and address the emergence and spread of MBL-producing gram-negative microorganisms in clinical environments.

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REFERENCES

1. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohtuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39:** 1612–1615.
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. **Bellais, S., O. Mimoz, S. Leotard, A. Jacolot, O. Petitjean, and P. Nordmann.** 2002. Efficacy of β-lactams for treating experimentally induced pneumonia due to a carbapenem-hydrolyzing metallo-β-lactamase-producing strain of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **46:**2032–2034.
4. **Bello, H., M. Dominguez, G. Gonzalez, R. Zemelman, S. Mella, H. K. Young, and S. G. Amyes.** 2000. In vitro activities of ampicillin, sulbactam and a combination of ampicillin and sulbactam against isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex isolated in Chile between 1990 and 1998. J. Antimicrob. Chemother. **45:**712–713.
5. **Bush, K., C. Macalintal, B. A. Rasmussen, V. J. Lee, and Y. Yang.** 1993. Kinetic interactions of tazobactam with β-lactamase from all major structural classes. Antimicrob. Agents Chemother. **37:**851–858.
6. **Campbell, M., E. Mahenthiralingam, and D. P. Speert.** 2000. Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **38:**4614–4615.
7. **Cardoso, O., R. Leitao, A. Figueiredo, J. C. Sousa, A. Duarte, and L. V. Peixe.** 2002. Metallo-β-lactamase VIM-2 in clinical isolates of *Pseudomonas aeruginosa* from Portugal. Microb. Drug Resist. **8:**93–97.
8. **Cornaglia, G., A. Mazzariol, L. Lauretti, G. M. Rossolini, and R. Fontana.** 2000. Hospital outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing VIM-1, a novel transferable metallo-β-lactamase. Clin. Infect. Dis. **31:**1119–1125.
9. **Docquier, J. D., J. Lamotte-Brasseur, M. Galleni, G. Amicosante, J. M. Frere, and G. M. Rossolini.** 2003. On functional and structural heterogeneity of VIM-type metallo-β-lactamases. J. Antimicrob. Chemother. **51:**257–266.
10. **Eliopoulos, G. M., K. Klimm, M. J. Ferraro, G. A. Jacoby, and R. C. Moellering.** 1989. Comparative in vitro activity of piperacillin combined with the β-lactamase inhibitor tazobactam (YTR 830). Diagn. Microbiol. Infect. Dis. **12:**481–488.
11. **Gales, A. C., L. C. Menezes, S. Silbert, and H. S. Sader.** 2003. Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-beta-lactamase. J. Antimicrob. Chemother. **52:**699–702.
12. **Hejazi, A., C. T. Keane, and F. R. Falkiner.** 1997. The use of RAPD-PCR as a typing method for *Serratia marcescens*. J. Clin. Microbiol. **46:**913–919.
13. **Hirakata, Y., K. Izumikawa, T. Yamaguchi, H. Takemura, H. Tanaka, R. Yoshida, J. Matsuda, M. Nakano, K. Tomono, S. Maesaki, M. Kaku, Y. Yamada, S. Kamihira, and S. Kohno.** 1998. Rapid detection and evaluation of clinical characteristics of emerging multiple-drug-resistant gram-negative rods carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **42:**2006–2011.
14. **Ito, H., Y. Arakawa, S. Ohsuka, R. Wacharotayankun, N. Kato, and M. Ohta.** 1995. Plasmid-mediated dissemination of the metallo-β-lactamase gene bla_{IMP} among clinically isolated strains of *Serratia marcescens*. Antimicrob. Agents Chemother. **39:**824–829.
15. **Iyobe, S., H. Kusadokoro, J. Ozaki, N. Matsumura, S. Minami, S. Haruta, T. Sawai, and K. O'Hara.** 2000. Amino acid substitutions in a variant of IMP-1 metallo-β-lactamase. Antimicrob. Agents Chemother. **44:**2023–2027.
16. **Iyobe, S., H. Kusadokoro, A. Takahashi, S. Yomoda, T. Okubo, A. Nakamura, and K. O'Hara.** 2002. Detection of a variant metallo-β-lactamase, IMP-10, from two unrelated strains of *Pseudomonas aeruginosa* and an *Alcaligenes xylosoxidans* strain. Antimicrob. Agents Chemother. **46:**2014–2016.
17. **Jones, R. N.** 1998. Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the Amp C enzymes. Diagn. Microbiol. Infect. Dis. **31:**461–466.
18. **Koh, T. H., G. S. Babini, N. Woodford, L. H. Sng, L. M. Hall, and D. Livermore.** 1999. Carbapenem-hydrolysing IMP-1 β-lactamase in *Klebsiella pneumoniae* from Singapore. Lancet **353:**2162.
19. **Kurokawa, H., T. Yagi, N. Shibata, K. Shibayama, and Y. Arakawa.** 1999. Worldwide proliferation of carbapenem-resistant gram-negative bacteria. Lancet **354:**955.
20. **Laraki, N., N. Franceschini, G. M. Rossolini, P. Santucci, C. Meunier, E. Pauw, G. Amicosante, J. M. Frere, and M. Galleni.** 1999. Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-β-lactamase IMP-1 produced by *Escherichia coli*. Antimicrob. Agents Chemother. **43:** 902–906.
21. **Lauretti, L., M. L. Riccio, A. Mazzariol, G. Cornaglia, G. Amicosante, R. Fontana, and G. M. Rossolini.** 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.
22. **Lee, K., J. B. Lim, J. H. Yum, D. Yong, Y. Chong, J. Kim, and D. M. Livermore.** 2002. bla_{VIM-2} cassette-containing novel integrons in metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolates disseminated in a Korean hospital. Antimicrob. Agents Chemother. **46:**1053–1058.
23. **Li, X. Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
24. **Matsumura, N., S. Minami, Y. Watanabe, S. Iyobe, and S. Mitsuhashi.** 1999. Role of permeability in the activities of β-lactams against gram-negative bacteria which produce a group 3 β-lactamase. Antimicrob. Agents Chemother. **43:**2084–2086.
25. **Mavroidi, A., A. Tsakris, E. Tzelepi, S. Pournaras, V. Loukova, and L. S. Tzouvelekis.** 2000. Carbapenem-hydrolysing VIM-2 metallo-β-lactamase in *Pseudomonas aeruginosa* from Greece. J. Antimicrob. Chemother. **46:**1041–1043.
26. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
27. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests, 6th ed. Approved standard M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
28. **National Committee for Clinical Laboratory Standards.** 2000. Performance standards for antimicrobial susceptibility testing. Tenth informational supplement, M100-S10. National Committee for Clinical Laboratory Standards, Wayne, Pa.
29. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. **37:**1661–1669.
30. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo-β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
31. **Ploy, M. C., F. Denis, P. Courvalin, and T. Lambert.** 2000. Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. Antimicrob. Agents Chemother. **44:**2684–2688.

32. **Poirel, L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J. D. Cavallo, and P. Nordmann.** 2000. Characterization of VIM2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob. Agents Chemother. 44:891–897.
33. **Riccio, M. L., N. Franceschini, L. Boschi, B. Caravelli, G. Cornaglia, R. Fontana, G. Amicosante, and G. M. Rossolini.** 2000. Characterization of the metallo-β-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of bla_{IMP} allelic variants carried by gene cassettes of different phylogeny. Antimicrob. Agents Chemother. 44:1229–1235.
34. **Sasaki, T., T. Nishiyama, M. Shintani, and T. Kenri.** 1997. Evaluation of a new method for identification of bacteria based on sequence homology of 16S rRNA gene. PDA J. Pharm. Sci. Technol. 51:242–247.
35. **Senda, K., Y. Arakawa, S. Ichiyama, K. Nakashima, H. Ito, S. Ohsuka, K. Shimokata, N. Kato, and M. Ohta.** 1996. PCR detection of metallo-β-lactamase gene (bla_{IMP}) in gram-negative rods resistant to broad-spectrum β-lactamases. J. Clin. Microbiol. 34:2909–2913.
36. **Senda, K., Y. Arakawa, K. Nakashima, H. Ito, S. Ichiyama, K. Shimokata, N. Kato, and M. Ohta.** 1996. Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum β-lactams, including carbapenems. Antimicrob. Agents Chemother. 40:349–353.
37. **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.
38. **Takahashi, A., S. Yomoda, I. Kobayashi, T. Okubo, M. Tsunoda, and S. Iyobe.** 2000. Detection of carbapenemase-producing *Acinetobacter baumannii* in a hospital. J. Clin. Microbiol. 38:526–529.
39. **Toleman, M. A., A. M. Simm, T. A. Murphy, A. C. Gales, D. J. Biedenbach, R. N. Jones, and T. R. Walsh.** 2002. Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance program. J. Antimicrob. Chemother. 50: 673–679.
40. **Tsakris, A., S. Pournaras, N. Woodford, M. F. Palepou, G. S. Babini, J. Douboyas, and D. M. Livermore.** 2000. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J. Clin. Microbiol. 38:1290–1292.
41. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19:6823–6831.
42. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 35:147–151.
43. **Woodford, N., M. F. Palepou, G. S. Babini, J. Bates, and D. M. Livermore.** 1998. Carbapenemase-producing *Pseudomonas aeruginosa* in UK. Lancet 352:546–547.
44. **Yamasaki, K., M. Komatsu, T. Yamashita, K. Shimakawa, T. Ura, H. Nishio, K. Satoh, R. Washidu, S. Kinoshita, and M. Aihara.** 2003. Production of CTX-M-3 extended-spectrum β-lactamase and IMP-1 metallo-β-lactamase by five gram-negative bacilli: survey of clinical isolates from seven laboratories collected in 1998 and 2000, in the Kinki region of Japan. J. Antimicrob. Chemother. 51:631–638.
45. **Yan, J. J., P. R. Hsueh, W. C. Ko, K. T. Luh, S. H. Tsai, H. M. Wu, and J. J. Wu.** 2001. Metallo-β-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. Antimicrob. Agents Chemother. 45:2224–2228.
46. **Yan, J. J., W. C. Ko, C. L. Chuang, and J. J. Wu.** 2002. Metallo-β-lactamase-producing *Enterobacteriaceae* isolates in a university hospital in Taiwan: prevalence of IMP-8 in *Enterobacter cloacae* and first identification of VIM-2 in *Citrobacter freundii*. J. Antimicrob. Chemother. 50:503–511.
47. **Yano, H., A. Kuga, R. Okamoto, H. Kitasato, T. Kobayashi, and M. Inoue.** 2001. Plasmid-encoded metallo-β-lactamase (IMP-6) conferring resistance to carbapenems, especially meropenem. Antimicrob. Agents Chemother. 45:1343–1348.

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Drug resistance of *Enterococcus faecium* clinical isolates and the conjugative transfer of gentamicin and erythromycin resistance traits

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Abstract

Drug resistance and the transferability of resistance were examined in 218 *Enterococcus faecium* clinical isolates obtained from inpatients of a Japanese university hospital between 1990 and 1999. One hundred and sixty one isolates (73.9%) were drug-resistant and 127 (58.2%) isolates were resistant to two or more drugs. Vancomycin resistant *E. faecium* (VRE) was not isolated. The transferability of drug-resistance to an *E. faecium* strain was examined by broth or filter mating. Six (12.5%) of the 48 gentamicin resistance traits, and fifty (50%) of the 101 erythromycin resistance traits were transferred by filter mating. The gentamicin resistance traits of five isolates and the erythromycin resistance traits of four isolates were transferred to the recipient strains by both broth mating and filter mating at a frequency of about 10^{-6} and 10^{-5} per donor cell, respectively. The five gentamicin resistant strains were shown to harbor pMG1-like plasmids on the basis of their Southern hybridization with pMG1 (65.1 kbp, Gm^r), which transfers efficiently between enterococci by broth mating. Each of the four erythromycin resistant transconjugants obtained by broth mating harbored a large conjugative plasmid (more than 100 kbp). The plasmids showed no homology with well-characterized enterococcal conjugative plasmids such as pAD1, pPD1, pAMβ1, pIP501 and pMG1 by Southern hybridization. Of the erythromycin resistance traits that transferred only by filter mating, it was found that the erythromycin resistance trait was conferred by a 47-kbp transposable element that transferred from the chromosome of the donor strain to different sites within the pheromone responsive plasmid pAD1 (60 kbp) of the recipient strain, suggesting that the erythromycin resistance trait was encoded on a conjugative transposon, which was named Tn*950*.

Keywords: *Enterococcus faecium*; Drug resistance; Conjugative plasmid; Conjugative transposon

1. Introduction

Enterococcus strains have become a significant cause of nosocomial infections and are the third most commonly isolated genus in clinical isolates [1–5]. Of the strains belonging to the enterococcus genus, *Enterococcus*

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faecalis and *Enterococcus faecium* are common isolates from human. The enterococcus strains isolated from clinical infection have multiple-drug resistance traits. The multiple-drug resistance traits of the enterococci provide these organisms with a selective advantage in the hospital environment. In particular, vancomycin (glycopeptide)-resistant enterococci (VRE or GRE) can cause serious problems for hospitalized patients due to the limited options for treatment of VRE infection [5–7].

Selective pressure by drugs or a genetic transfer system is essential for the increase or spread of drug resistant organisms. *E. faecium* clinical isolates are usually multiple-resistant and VanA type vancomycin resistant enterococci (VRE) are predominately isolated from *E. faecium* [6,7]. Transferable plasmids or mobile genetic elements encoding drug resistance traits have been reported in *E. faecium*. The VanA-type resistance determinant is encoded on transposon Tn*1546*, which is borne by non-conjugative or conjugative plasmids that transfer among enterococci by mating on a solid surface (filter mating) [8–10]. The VanB determinant of *E. faecium* is encoded on a large mobile genetic element on a conjugative transposon [11–13].

Little is known about the systems of efficient plasmid transfer in *E. faecium*. Previously, we reported the first isolation of a new type of conjugative plasmid, named pMG1 [14–16], which transfers efficiently among enterococcus strains during broth mating, carries gentamicin resistance trait, and was isolated from an *E. faecium* clinical isolate in Japan. Conjugative gentamicin resistance pMG1-like plasmids are widely disseminated in vancomycin resistant *E. faecium* clinical isolates in the US [15], and are thought to contribute to the spread of other resistance traits, including vancomycin resistance in enterococci [15,16]. In this report, we describe the drug resistance of *E. faecium* clinical isolates from a Japanese hospital, and the transferabilities of the drug resistance traits.

2. Materials and methods

2.1. Bacteria, plasmid and media

A total of 218 *E. faecium* clinical isolates were used in this study. These isolates were obtained from different patients who had been admitted to Gunma University Hospital, Maebashi, Japan, between January 1990 and December 1999. A total of 41 *E. faecium* isolates from the feces of different healthy students were used as control strains. The laboratory strains and plasmids used in this study were *E. faecalis* FA2-2 (Rif^r, Fus^r) [17], JH2SS (Str^r, Spc^r) [18], *E. faecium* BM4105RF (Rif^r, Fus^r) [15], BM4105SS (Str^r, Spc^r) [15], plasmid pMG1 (gentamicin resistances) (65.1 kbp) [14], pAD1 [19–25], pPD1 [26–28], pAM373 [29], pAMβ1 [30], pIP501 [31,32] and pAM120 [33]. The media used in this study were Todd-Hewitt broth (THB) and agar plates (Difco Laboratories, Detroit, Mich.). The MIC of the antimicrobial drugs was determined according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) using Mueller-Hinton agar. The antibiotic concentrations used in the selective plates were as follows (in micrograms per milliliter): ampicillin, 25; fosfomycin, 25; erythromycin, 25; streptomycin, 500; spectinomycin, 500; kanamycin, 500; gentamicin, 500; chloramphenicol, 25; tetracycline, 3; vancomycin, 3; rifampin, 25; and fusidic acid, 25.

2.2. Mating procedures

Broth mating and filter mating were performed as previously described [34–36] with a donor/recipient ratio of 1:10.

2.3. Isolation and manipulation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method [37,38].

2.4. Pulsed-field gel electrophoresis of chromosomal DNA

Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA isolated from the *E. faecium* strain was performed as previously described [3]. The gels were electrophoresed with a clamped homogeneous electric field (CHEF-DR II; Bio-Rad Laboratories, Richmond, CA).

2.5. Southern hybridization

Southern hybridization was performed with the digoxigenin-based non-radioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols [15,37].

3. Results and discussion

3.1. Drug resistance and the conjugative transfer of high-level erythromycin and gentamicin resistance

As shown in Table 1, 161 (73.9%) isolates were drug resistant and the remaining 57 (26.1%) isolates were drug susceptible. Tetracycline, erythromycin, and ampicillin resistant strains were isolated at relatively high frequencies of about 50–40% when compared to other drug resistances, and 48 (22.0%) of the isolates had a high level of resistance to gentamicin (more than 500 μg/ml MIC). No vancomycin resistant isolates were obtained.

Table 1
Isolation of drug resistance of *E. faecium*

Drug resistance		Number of drug resistant strain (%)	
		Clinical isolates	Isolates from feces of healthy students
Ampicillin	(Ap)	97 (44.5%)	
Chloramphenicol	(Cm)	8 (3.7%)	
Erythromycin	(Em)	101 (46.3%)	1 (2.4%)
Gentamicin	(Gm)	48 (22.0%)	1 (2.4%)
Kanamycin	(Km)	82 (37.6%)	3 (7.3%)
Streptomycin	(Sm)	41 (18.8%)	1 (2.4%)
Tetracyclin	(Tc)	120 (55.0%)	5 (12.2%)
Vancomycin	(Vm)	0 (0%)	
Drug susceptible		57 (26.1%)	33 (80.5%)
Total number of strains tested		218 (100%)	41 (100%)

The drug resistance levels (MICs) of ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracyclin, and vancomycin were equal to or greater than 16, 16, 16, 64, 1024, 512, 8 and 64 μg/ml, respectively.

Drug resistance among the isolates showed many different patterns and there was no predominant drug resistant pattern. Approximately 30% of the isolates were resistant to more than four drugs (data not shown).

To examine the transferability of the high-level erythromycin (more than 100 μg/ml MIC) and gentamicin resistances, broth mating or filter mating experiments were performed between each drug resistant isolate and the recipient strain *E. faecium* BM4105RF. Of the 48 gentamicin resistant isolates, five were transferred by broth mating and filter mating, and one was transferred by filter mating only. Of the 101 erythromycin resistant isolates, four were transferred by broth mating and filter mating, and 46 were transferred only by filter mating.

3.2. Gentamicin resistance conjugative plasmid

Each of the five gentamicin resistant strains, which were obtained by broth mating, were designated as GF112, GF113, EFG13, EFG16, and EFG17 and their drug resistance patterns were Em Gm Km Sm Tc, Ap Gm Sm Tc, Ap Em Gm Km Sm Tc, Ap Gm Km Sm Tc, and Ap Em Gm Km Sm Tc, respectively. Each gentamicin resistance trait was transferred to an *E. faecium* or *E. faecalis* strain at a frequency of 10^{-5}–10^{-7} per donor cell by broth mating or more than 10^{0} per donor cell by filter mating. The transfer frequency more than 10^{0} per donor cell resulted from that plasmid of all donor cells could transfer to recipient cell and plasmid of the transconjugant could re-transfer to recipient cell. The resistance of ten transconjugants of each strain was examined in detail. All of the transconjugants were resistant only to gentamicin. The gentamicin resistance conjugative plasmid pMG1 (65.1 kbp) was originally isolated from *E. faecium* GF113 [14]. The plasmids isolated from GF112, EFG13, EFG16, and EFG17 exhibited *Eco*RI restriction profiles almost identical to pMG1

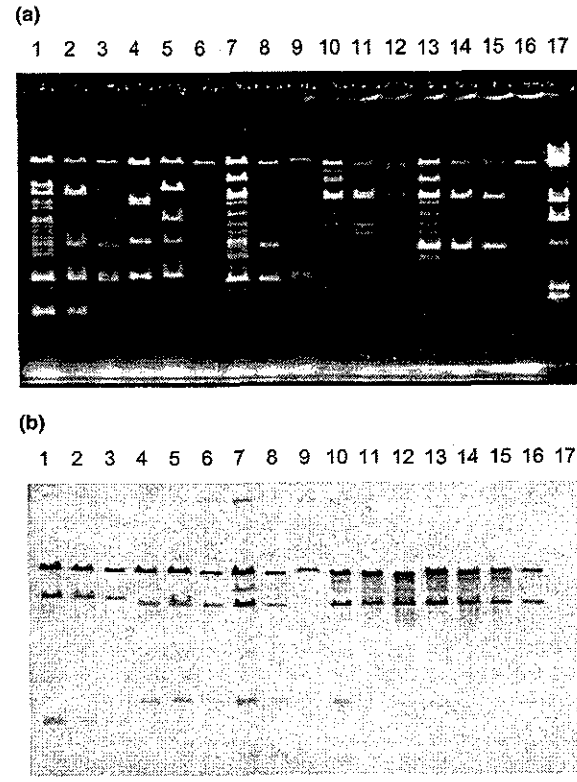

Fig. 1. Agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs of *E. faecium* isolates harboring a pMG1-like plasmid, and Southern hybridization with pMG1. (a) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from gentamicin-resistant *E. faecium* clinical isolates, the gentamicin-resistant *E. faecium* BM4105RF transconjugants and *E. faecalis* FA2-2 transconjugants. (b) The gel was Southern blotted and hybridized to pMG1. Lanes 1, 4, 7, 10, 13: *Eco*RI-digested plasmid DNA isolated from the clinical isolates GF112, GF113, EFG13, EFG16, and EFG17, respectively; lanes 2, 5, 8, 11, 14: *Eco*RI-digested plasmid DNA isolated from BM4105RF transconjugants of strains GF112, GF113, EFG13, EFG16, and EFG17, respectively; lanes 3, 6, 9, 12, 15: *Eco*RI-digested plasmid DNA isolated from FA2-2 transconjugants of strains GF112, GF113, EFG13, EFG16, and EFG17, respectively; lane 16: *Eco*RI-digested pMG1; lane 17: *Hin*dIII-digested lambda DNA.

and hybridized to pMG1 DNA (Fig. 1). These data implied that these isolates harbored pMG1-like plasmids.

Analysis by pulsed field gel electrophoresis of *Sma*I-digested chromosomal DNA from GF112, GF113, EFG13, EFG16 and EFG17 showed four different patterns (data not shown). Although the isolation frequency of gentamicin resistant strains harboring pMG1-like plasmids was still low, the pMG1-like plasmid could also disseminated to different strains in Japanese clinical isolates. EFG13 and EFG17 had identical restriction endonuclease digestion patterns. EFG13 and EFG17 were isolated from patients in the same ward who had overlapping periods of hospitalization, which implied that nosocomial transmission of the strain had occurred (data not shown).

3.3. Highly efficient transfer of high-level erythromycin resistance and the conjugative plasmid

There was a possibility that each of the four strains designated as EFG114, EFG115, EFG132 and EFG160 that transferred the high-level erythromycin resistance by broth mating were harboring the erythromycin resistance transferable plasmid. Each erythromycin resistance trait was transferred at a frequency of around 10^{-4}–10^{-5} per donor cell in broth, or around 10^{-2}–10^{-1} per donor cell in filter mating between *E. faecium* BM4105 strains.

The plasmid DNA was prepared from each erythromycin resistant (Em^r) transconjugant of BM4105RF and analyzed by agarose gel electrophoresis. Agarose gel electrophoresis produced many faint bands of *Eco*RI restriction fragments of each plasmid, which implied that the molecular sizes of the plasmid DNAs were relatively large and these were estimated to be more than 100 kbp (Fig. 2(a)). It is probable that the conjugative erythromycin resistance plasmids are related to each other with respect to their DNA similarity. The plasmid DNA isolated from the EFG132 transconjugant was labeled for use as a probe in Southern hybridization (Fig. 2(b)). The plasmid DNAs isolated from each of Em^r transconjugants showed a similar *Eco*RI restriction profile with respect to the DNA fragments that hybridized to the EFG132 plasmid DNAs by Southern analysis. In particular, two plasmids isolated from the transconjugants of EFG132 and EFG160 showed an identical *Eco*RI restriction profile. The plasmid DNAs of the EFG132 transconjugant showed no homology with pMG1 (Fig. 2, lane 5). Southern analysis was also performed using the plasmid DNAs of the EFG132 transconjugant and the pheromone-responsive plasmids pAD1, pPD1, broad-host-range conjugative plasmids pAMβ1, pIP501, and conjugative transposon Tn*916*. It showed no homology with other plasmids or the conjugative transposon (data not shown), indicating that the plasmids might be another type of *E. faecium* conjugative plasmids. The high-level erythromycin resistances (more than 100 μg/ml MIC) of *E. faecium* are generally determined by *erm* genes such as *ermB* and *ermA* [39]. The genes encode 23S rRNA methyl transferase and locate on the mobile elements such as transposon.

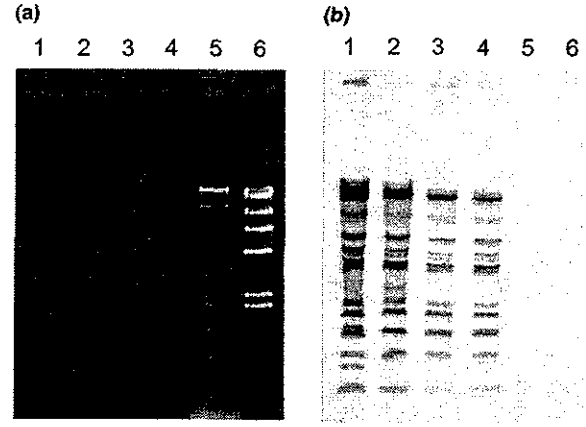

Fig. 2. Agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs of erythromycin resistant *E. faecium* BM4105RF transconjugants isolated from erythromycin resistant *E. faecium* isolates, and Southern hybridization with the erythromycin-resistant plasmid of EFG132. (a) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from erythromycin-resistant BM4105RF transconjugants isolated from erythromycin resistant isolates. (b) The gel was Southern blotted and hybridized with the plasmid DNA isolated from the transconjugant of EFG132. Lanes 1–4: *Eco*RI-digested plasmid DNAs isolated from the transconjugants of EFG114, EFG115, EFG132, EFG160, respectively; lane 5: *Eco*RI-digested pMG1; lane 6: *Hin*dIII-digested lambda DNA.

3.4. Conjugative transfer of chromosome borne high-level erythromycin resistance

The erythromycin resistance of *E. faecium* T383 was transferred to the recipient strains *E. faecalis* FA2-2 and *Enterococcus hirae* ATCC9790RF at a frequency of 8.6×10^{-8} and 5.7×10^{-6} per donor cell by filter mating, respectively. The erythromycin resistance of each of the transconjugants was transferred between *E. faecalis* strains, between *E. hirae* and *E. faecalis* strains, and between *E. hirae* strains by filter mating at a frequency of approximately 10^{-4}–10^{-5} per donor cell. The parent strain *E. faecium* T383 contained several plasmids (data not shown). Of the erythromycin resistant *E. faecalis* FA2-2 transconjugants, one transconjugant that was named *E. faecalis* KT1 was found to be devoid of plasmid after repeated transfer experiments. The erythromycin resistance of *E. faecalis* KT1 was also transferred among enterococcus strains during filter mating at a frequency of around 10^{-5}–10^{-6} per donor cell. These results implied that the erythromycin resistance determinant might reside on a transposable element that is located on the bacterial chromosome and is capable of subsequent transfer by filter mating.

The plasmid pAD1 (hemolysin/bacteriocin, 60 kbp) is an *E. faecalis* pheromone-responsive plasmid that transfers at high frequency to the *E. faecalis* recipient strain during broth mating at a frequency 10^{-2}–10^{0} [17,20]. Filter mating experiments were performed using the erythromycin resistant transconjugant of *E. faecalis* JH2SS as a donor and *E. faecalis* FA2-2 harboring pAD1 plasmid as a recipient. Erythromycin resistant transconjugants of *E. faecalis* FA2-2 (pAD1) were obtained at a frequency of around 10^{-5} per donor cell. If the erythromycin resistance transposable element was transposed to pAD1 and was linked with the plasmid, the resistance would be transferred to the recipient *E. faecalis* strain at a relatively high frequency as the pheromone responsive plasmid pAD1 in subsequent broth mating experiments. About 10% of the erythromycin resistant *E. faecalis* FA2-2 transconjugants was found to transfer erythromycin resistance to the recipient JH2SS strains at the relatively high frequency of 10^{-2} per donor cell by broth mating, indicating linkage of the resistance determinant to the plasmid. Four erythromycin resistant *E. faecalis* FA2-2 (pAD1) transconjugants, which transferred the erythromycin resistance at relatively high frequency by subsequent broth mating, were chosen as representative strains.

3.5. Agarose gel electrophoresis analysis of pAD1 borne erythromycin resistance

The agarose gel electrophoresis profile of *Eco*RI-digested plasmid DNA from each of the four strains differed from that of pAD1, which consists of nine *Eco*RI fragments designated A to I with corresponding molecular sizes of 19.3, 15.4, 12.0, 4.1, 3.4, 2.7, 1.5, 1.2 and 0.2 kbp (Fig. 3(a)). Of the plasmids isolated from the four strains, one plasmid (pMG3001, Fig. 3(a), lane 3) did not have the 1.2 kbp fragment corresponding to *Eco*RI fragment H of pAD1 and the other three

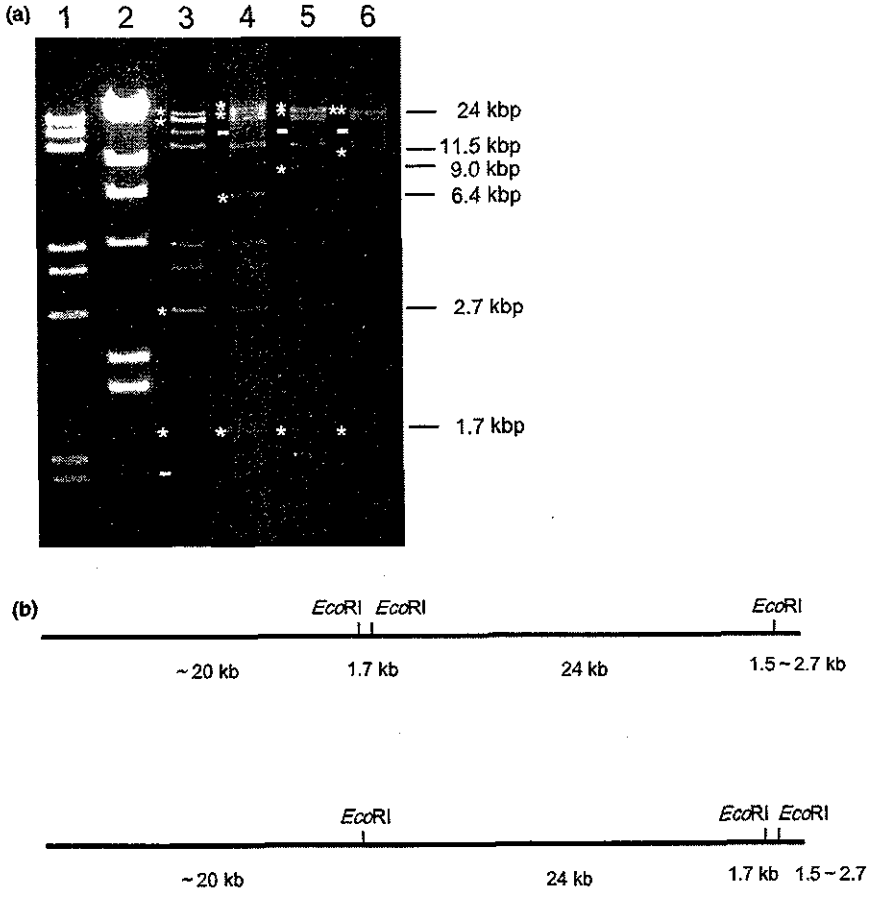

Fig. 3. Agarose gel electrophoresis of *Eco*RI-digested DNAs of pAD1 borne erythromycin resistance and the possible structure of the erythromycin resistance transposable element (Tn*950*). (a) *Eco*RI-digested plasmid DNA isolated from erythromycin-resistance pAD1. Lane 1: *Eco*RI digested pAD1; lane 2: *Hin*dIII-digested lambda DNA; lanes 3–6: *Eco*RI-digested plasmid DNAs of pMG3001, pMG3002, pMG3003 and pMG3004, respectively. Symbols: the star and small bar on the left side of the agarose gel for each plasmid indicates the new band and missing band, respectively. (b) Two putative physical maps of the erythromycin resistance 47 kbp transposable element (Tn*950*). There are three *Eco*RI sites in the transposon and two internal *Eco*RI fragments (24 and 1.7 kbp).

plasmids (i.e., pMG3002 (lane 4), pMG3003 (lane 5), pMG3004 (lane 6)) did not have the 15.4 kbp fragment corresponding *Eco*RI fragment B of pAD1. Each of four plasmids gave rise to four new fragments. The molecular sizes of the new fragments of pMG3001 were 24, 20, 2.7 and 1.7 kbp. The total molecular size of the four new fragments of pMG3001 was about 48.4 kbp. These results implied that *Eco*RI fragment H (1.2 kbp) of pAD1 had an insertion with molecular size of about 47 kbp, and this 48.2 kbp fragment gave rise to new four *Eco*RI fragments. The molecular sizes of the new fragments of pMG3002 were 30, 24, 6.4 and 1.7 kbp (Fig. 3(a), lane 4). The molecular sizes of the new fragments of pMG3003 were 28, 24, 9.0 and 1.7 kbp (Fig. 3(a), lane 5). The molecular sizes of the new fragment of pMG3004 were 25, 24, 11.5 and 1.7 kbp (Fig. 3(a), lane 6). The total molecular size of the four new fragments of each of these plasmids was about 62.5 kbp. These data indicated that *Eco*RI fragment B (15.4 kbp) of pAD1 had an insertion with molecular size of about 47 kbp, and that the 62.5 kbp fragment gave rise to new four *Eco*RI fragments in each of the three plasmids. Of the four new *Eco*RI fragments observed in each plasmid, two fragments with a molecular size of 24 and 1.7 kbp were found in each of the four plasmids. The other two *Eco*RI fragments varied in size depending on which of the four plasmids they originated from, indicating that each of the two fragments contained either one fragment that separated *Eco*RI fragment H (1.2 kbp) of pAD1 by insertion of the 47 kbp element in case of pMG3001, and separated *Eco*RI fragment B (15.4 kbp) of pAD1 by insertion of the 47 kbp element in case of pMG3002, pMG3003 and pMG3004, respectively. These results implied that the approximately 47 kbp element encoding erythromycin resistance had transposed from the chromosomal DNA to different sites within pAD1 by conjugation. The 47 kbp element, which will be subsequently referred to as conjugative transposon Tn*950*, had three *Eco*RI sites and the two internal *Eco*RI fragments were 1.7 and 24 kbp in size. The putative *Eco*RI sites of Tn*950* are shown in Fig. 3(b).

3.6. DNA–DNA hybridization

The conjugative transposon Tn*916* (16 kbp, *tet*M) was originally isolated from *E. faecalis* DS16 and is a representative conjugative transposon. The transposon Tn*917* (5 kbp, MLS^r (macrolide-limcosamide-streptogramin B resistance)) was also originally isolated from *E. faecalis* DS16 and is a member of the Tn*3* family. Tn*917* encodes *ermB* gene conferring the high-level erythromycin resistance and Tn*917*-like transposon is widespread in enterococcus species. The plasmid pMG3005, that is another pAD1 derivative carrying an erythromycin resistance transposable element within the *Eco*RI fragment B, was studied for homology with Tn*917* and Tn*916*. pAM225 is the pBR332-derived vector pBR325 carrying the pAD1 *Eco*RI fragment H::Tn*917*. pAM120 is the pBR322-derived vector pGL101 carrying the pAD1 *Eco*RI fragment F::Tn*916*. The plasmids pAM225 and pAM120 were used as probes for Tn*917* and Tn*916*, respectively. pAM120 only hybridized to a 2.7 kbp *Eco*RI fragment of pMG3005 corresponding to *Eco*RI fragment F of pAD1, which is contained in pAM210 (Fig. 4(b)). These results suggested that the erythromycin resistance transposable element did not contain any sequence homologous with Tn*916*. pAM225 hybridized to a 1.2-kbp *Eco*RI fragment of pMG3005 corresponding to *Eco*RI fragment H of pAD1, which is contained in pAM225, and also hybridized to the 24-kbp *Eco*RI fragment of pMG3005, which corresponds to the new *Eco*RI fragment of pMG3005, and the hybridization band was relatively faint (Fig. 4(c)). The results suggested that the erythromycin resistance transposable element did not contain significant sequence homologous with Tn*917* and the faint signal might indicate the similarity with the erythromycin resistance determinant (*ermB* gene) encoded on the transposon.

Conjugative transposons are genetic elements that move from the genome of a donor cell to the genome of a recipient bacterial cell by conjugation with cell to cell contact [36,40,41]. Conjugative transposons are widespread in gram-positive bacteria and play a significant role in the dissemination of antibiotic resistance and are particularly common in streptococci and enterococci [42]. Tn*916* (18kbp) and Tn*1545* (25.3 kbp) from

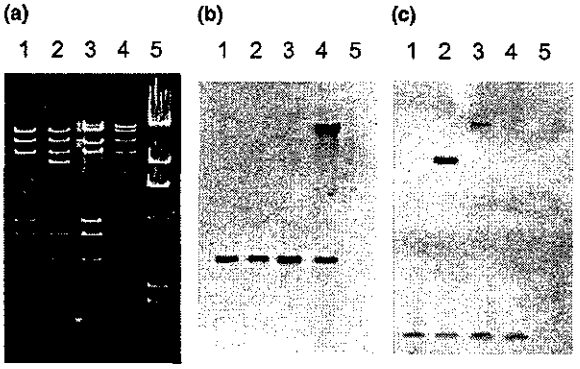

Fig. 4. Southern hybridization analysis of the 47 kbp transposable element (Tn*950*) that carries erythromycin resistance with the Tn*916* probe (b) and Tn*917* probe (c). (a) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNAs of pAD1 and the derivatives. Symbols: the star and small bar on the left side of the agarose gel of lane 3 indicates the new band and missing band, respectively. (b) Southern hybridization analysis using the labeled whole plasmid DNA of pAM120 as a Tn*916* probe. (c) Southern hybridization analysis using the labeled whole plasmid DNA of pAM225 as a Tn*917* probe. Lanes 1: pAD1; lane 2: pAM307 (pAD1::Tn*917*); lane 3: pGM3005 (pAD1::Tn*950*, Tn*950* inserted into *Eco*RI fragment B of pAD1); lane 4: pAM210 (pAD1::Tn*916*); lane 5: *Hin*dIII-digested lambda DNA.

E. faecalis and *Streptococcus pneumoniae*, respectively, are the best-studied examples of conjugative transposons in gram-positive bacteria and are representative of the conjugative transposons. They and many other conjugative transposons confer tetracycline resistance on their host strains by the *tet*M determinant that encodes a ribosome binding protein [42]. The conjugative transposons are excised and integrated via a reciprocal recombination mechanism using the Int and Xis proteins, which belong to lambda integrase family and are analogous to lambda Xis protein, respectively.

The 47 kbp transposable element did not contain any sequence homologous with that of the representative conjugative transposon Tn*916*. Although the nature of intercellular transfer of the 47 kbp element was unknown, these data implied that the 47 kbp element might be different type of conjugative transposon from the Tn*916*-like transposon.

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References

[1] Arakawa, Y., Ike, Y., Nagasawa, M., Shibata, N., Doi, Y., Shibayama, K., Yagi, T. and Kurata, T. (2000) Trends in antimicrobial-drug resistance in Japan. Emerg. Infect. Dis. 6, 572–575.

[2] Murray, B.E. (1990) The life and times of the enterococcus. Clin. Microbiol. Rev. 3, 46–65.

[3] Ma, X., Kudo, M., Takahashi, A., Tanimoto, K. and Ike, Y. (1998) Evidence of nosocomial infection in Japan caused by high-level gentamicin-resistant *Enterococcus faecalis* and identification of the pheromone-responsive conjugative plasmid encoding gentamicin resistance. J. Clin. Microbiol. 36, 2460–2464.

[4] Martone, W.J. (1998) Spread of vancomycin resistant enterococci: why did it happen in the United States? Infect. Control Hosp. Epidemiol 19, 539–545.

[5] Uttley, A.H., Collins, C.H., Naidoo, J. and George, R.C. (1988) Vancomycin-resistant enterococci. Lancet I, 57–58.

[6] Huycke, M.M., Sahm, D.F. and Gilmore, M.S. (1998) Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. 4, 239–249.

[7] Martone, W.J. (1998) Spread of vancomycin resistant enterococci: why did it happen in the United States. Infect. Control Hosp. Epidemiol. 19, 539–545.

[8] Arthur, M., Molinas, C., Depardieu, F. and Couvalin, P. (1993) Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. 175, 117–127.

[9] Leclercq, R., Derlot, E., Weber, M., Duval, J. and Courvalin, P. (1988) Plasmid-mediated resistance to vancomycin and teicoplamin in *Enterococcus faecium*. N. Engl. J. Med. 319, 157–161.

[10] Leclercq, R., Derlot, E., Duval, J. and Courvalin, P. (1989) Transferable vancomycin and teicoplanin resistance in *Enterococcus feacium*. Antimicrob. Agents. Chemother. 33, 10–15.

[11] Quintiliani Jr., R. and Courvalin, P. (1996) Characterization of Tn*1547*, a composite transposon flanked by the IS*16* and IS*256*-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene 172, 1–8.

[12] Garnier, F., Taourit, S., Glaser, P., Courvalin, P. and Galimand, M. (2000) Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp.. Microbiology 146, 1481–1489.

[13] Carias, L.L., Rudin, S.D., Donskey, D.J. and Rice, L.B. (1998) Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J. Bacteriol. 180, 4426–4434.

[14] Ike, Y., Tanimoto, K., Tomita, H., Takeuchi, K. and Fujimoto, S. (1998) Efficient transfer of the pheromone-independent *Enterococcus faecium* plasmid pMG1 (Gm^r) (65.1 kilobases) to *Enterococcus* strains during broth mating. J. Bacteriol. 180, 4886–4892.

[15] Tomita, H., Pierson, C., Lim, S.K., Clewell, D.B. and Ike, Y. (2002) Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. J. Clin. Microbiol. 40, 3326–3333.

[16] Tomita, H., Tanimoto, K., Hayakawa, S., Morinaga, K., Ezaki, K., Oshima, H. and Ike, Y. (2003) A highly efficient pMG1-like conjugative plasmid mediates the VanA resistance encoded on a Tn *1546*-like element in *Enterococcus faecium*. J. Bacteriol. 185, 7024–7028.

[17] Clewell, D.B., Tomich, P.K., Gawron-Burke, M.C., Franke, A.E., Yagi, Y. and An, F.Y. (1982) Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. 152, 1220–1230.

[18] Tomich, P.K., An, F.Y. and Clewell, D.B. (1980) Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. 141, 1366–1374.

[19] Clewell, D.B. (1993) Bacterial sex pheromone-induced plasmid transfer. Cell 73, 9–12.

[20] Clewell, D.B. (1993) Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis* In: Bacterial Conjugation (Clewell, D.B., Ed.), pp. 349–367. Plenum Press, New York, NY.

[21] Clewell, D.B., Francia, M.V., Flannagan, S.E. and An, F.Y. (2002) Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. Plasmid 48, 193–201.

[22] Ike, Y., Craig, R.A., White, B.A., Yagi, Y. and Clewell, D.B. (1983) Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. USA 80, 5369–5373.

[23] Fujimoto, S. and Clewell, D.B. (1998) Regulation of the pAD1 sex pheromone response of *Enterococcus faecalis* by direct interaction between the cAD1 peptide mating signal and the negatively regulating, DNA-binding TraA protein. Proc. Natl. Acad. Sci. USA 95, 6430–6435.

[24] Tanimoto, K. and Clewell, D.B. (1993) Regulation of the pAD1-encoded sex pheromone response in *Enterococcus faecalis*: expression of the positive regulator TraE1. J. Bacteriol. 175, 1008–1018.

[25] Tomita, H. and Clewell, D.B. (2000) A pAD1-encoded small RNA molecule, MD, negatively regulates *Enterococcus faecalis* pherome response by enhancing transcription termination. J. Bacteriol. 182, 1062–1073.

[26] Yagi, Y., Kessler, R., Shaw, J., Lopatin, D.E., An, F. and Clewell, D.B. (1983) Plasmid content of *Streptococcus faecalis* strain 39-5 and identification of a pheromone (cPD1)-induced surface antigen. J. Gen. Microbiol. 129, 1207–1215.
[27] Fujimoto, S., Tomita, H., Wakamatsu, K., Tanimoto, K. and Ike, Y. (1995) Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J. Bacteriol. 177, 5574–5581.
[28] Tomita, H., Fujimoto, S., Tanimoto, K. and Ike, Y. (1997) Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid. J. Bacteriol. 179, 7843–7855.
[29] Clewell, D.B., An, F.Y., White, B.A. and Gawron-Burke, C. (1985) *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn*918*). J. Bacteriol. 161, 1212–1220.
[30] Leblanc, D.L. and Lee, L.N. (1984) Physical and genetic analysis of streptococcal plasmid pAMβ1 and cloning of its replication region. J. Bacteriol. 157, 445–453.
[31] Evans, R.P. and Macrina, F.L. (1983) Streptococcal R plasmid pIP501: endonuclease site map, resistance determinant location, and construction of novel derivatives. J. Bacteriol. 154, 1347–1355.
[32] Krah III, E.R. and Macrina, F.L. (1989) Genetic analysis of the conjugal transfer determinants encoded by the streptococcal broad-host-range plasmid pIP501. J. Bacteriol. 171, 6005–6012.
[33] Gawron-Burke, C. and Clewell, D.B. (1984) Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escheria coli*: strategy for targeting and cloning of genes from gram-positive bacteria. J. Bacteriol. 159, 214–221.
[34] Dunny, G.M., Craig, R.A., Carron, R.L. and Clewell, D.B. (1979) Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. Plasmid 2, 454–465.
[35] Ike, Y. and Clewell, D.B. (1984) Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. 158, 777–783.
[36] Franke, A.E. and Clewell, D.B. (1980) Evidence for conjugal transfer of a *Streptococcus faecalis* transposon (Tn*916*) from a chromosomal site in the absence of plasmid DNA. Cold Spring Harbor Symp. Quant. Biol. 45, 77–80.
[37] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[38] Tomita, H., Fujimoto, S., Tanimoto, K. and Ike, Y. (1996) Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. 178, 3585–3593.
[39] Portillo, A., Ruiz-Larrea, F., Zarazaga, M., Alonso, A., Martinez, J.L. and Torres, C. (2000) Macrolide resistance genes in *Enterococcus* spp.. Antimicrob. Agents. Chemother. 44, 967–971.
[40] Clewell, D.B. (1981) Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. 45, 409–436.
[41] Clewell, D.B. and Gawron-Burke, C. (1986) Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu. Rev. Microbiol. 40, 635–659.
[42] Clewell, D.B., Flannagan, S.E. and Jaworski, D.D. (1995) Unconstrained bacterial promiscuity: the Tn*916*–Tn*1545* family of conjugative transposons. Trends Microbiol. 3, 229–236.

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CFE-1, a Novel Plasmid-Encoded AmpC β-Lactamase with an *ampR* Gene Originating from *Citrobacter freundii*

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A clinical isolate of *Escherichia coli* from a patient in Japan, isolate KU6400, was found to produce a plasmid-encoded β-lactamase that conferred resistance to extended-spectrum cephalosporins and cephamycins. Resistance arising from production of a β-lactamase could be transferred by either conjugation or transformation with plasmid pKU601 into *E. coli* ML4947. The substrate and inhibition profiles of this enzyme resembled those of the AmpC β-lactamase. The resistance gene of pKU601, which was cloned and expressed in *E. coli*, proved to contain an open reading frame showing 99.8% DNA sequence identity with the *ampC* gene of *Citrobacter freundii* GC3. DNA sequence analysis also identified a gene upstream of *ampC* whose sequence was 99.0% identical to the *ampR* gene from *C. freundii* GC3. In addition, a fumarate operon (*frdABCD*) and an outer membrane lipoprotein (*blc*) surrounding the *ampR-ampC* genes in *C. freundii* were identified, and insertion sequence (IS*26*) elements were observed on both sides of the sequences identified (forming an IS*26* composite transposon); these results confirm the evidence of the translocation of a β-lactamase-associated gene region from the chromosome to a plasmid. Finally, we describe a novel plasmid-encoded AmpC β-lactamase, CFE-1, with an *ampR* gene derived from *C. freundii*.

The AmpC β-lactamase produced by gram-negative bacteria such as *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Morganella* spp. can hydrolyze several β-lactam antibiotics, including cephamycins and extended broad-spectrum cephalosporins (30).

The regulation of AmpC β-lactamase expression is intimately linked to cell wall recycling and involves at least three genes: *ampR*, which encodes a transcriptional regulator of the LysR family; *ampG*, which encodes a transmembrane permease; and *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that hydrolyzes 1,6-anhydromuropeptides (16, 21, 23, 31). AmpR has been shown to bind to a 38-bp sequence within the intercistronic region between *ampR* and *ampC*. In the absence of a β-lactam inducer, AmpR represses the synthesis of β-lactamase 2.5-fold, whereas expression is induced 10- to 200-fold in the presence of a β-lactam inducer (26). Mutations in the specific site of *ampR* work as an activator of *ampC* and result in the constitutive hyperproduction of AmpC β-lactamase (3, 4, 24). Deletion mutation of the *ampR* gene results in a slightly higher level of basal expression of the *Citrobacter freundii* β-lactamase, but enzyme synthesis can no longer be induced. Knockout mutations in the *ampD* gene result in constitutive hyperproduction of the AmpC β-lactamase even in the absence of a β-lactam inducer (6, 18).

In recent years *ampC* genes have been found on conjugative plasmids, mainly among *Klebsiella pneumoniae* isolates but also occasionally among *Escherichia coli* isolates. Some of these plasmid-encoded genes have DNA and amino acid sequences very similar to those of the chromosome-encoded AmpC β-lactamases of *C. freundii* (CMY-2, CMY-4, and LAT-1) (1, 5, 42, 43), *Enterobacter cloacae* (MIR-1 and ACT-1) (11, 33), and *Morganella morganii* (DHA-1 and DHA-2) (13, 14), although the phylogenies of the various enzymes (FOX-1, MOX-1, and CMY-9) (12, 15, 19) remain unclear (Fig. 1).

Until recently, plasmid-encoded *ampC* genes were considered noninducible because they lack the regulator gene *ampR* (35). However, this generalization is no longer valid: three inducible plasmid-encoded AmpC β-lactamases, DHA-1, DHA-2, and ACT-1, have been described; and all of these carry the *ampR* and the *ampC* genes (2, 13, 37). The mechanism by which plasmid-encoded AmpC β-lactamase was generated from the chromosomal gene has not yet been discovered.

Compared with plasmid-encoded class A extended-spectrum β-lactamases, these plasmid-encoded AmpC β-lactamases (except for ACC-1) are active against cephamycins and are also effective against oxyimino-cephalosporins, such as cefotaxime, ceftazidime, and aztreonam, a monobactam. The in vitro activities of these AmpC β-lactamases are not inhibited by clavulanic acid. Genes encoding these enzymes are now found on plasmids at increasing frequencies (34).

A plasmid-encoded AmpC β-lactamase which confers resistance to cephamycins and expanded-spectrum cephalosporins was detected in Japan in a clinical isolate of *E. coli*. In this report, we characterize a novel plasmid-encoded AmpC β-lactamase, CFE-1, and analyze the nucleotide sequences of the *ampC*, *ampR*, and surrounding genes to compare with those of chromosome-encoded and plasmid-encoded AmpC β-lactamases.

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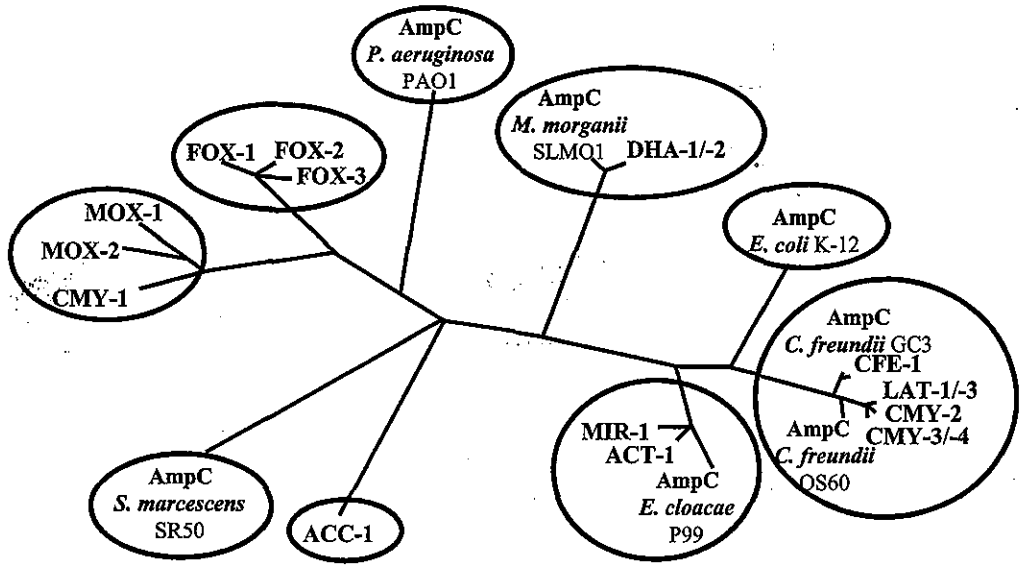

FIG. 1. Dendrogram for chromosomal and plasmid-encoded AmpC β-lactamases calculated by the Clustal V program by using the neighbor-joining method (38).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Clinical strain *E. coli* KU6400 was isolated at a hospital in Japan in 1997 and was found to contain plasmid pKU601. *E. coli* K-12 ML4947 (AmpD wild type) and ML4953 (AmpD mutant) strains were used as recipients of the plasmid (24, 27). Plasmid pHSG398 is a vector plasmid that confers resistance to chloramphenicol.

Antibacterial agents. Reference samples of the antibacterial agents listed below were used in this study and were provided as powders of known potencies by the respective manufacturers. Piperacillin (Toyama Chemical, Toyama, Japan) was used as a representative penicillin, while cephalothin (Shionogi, Osaka, Japan), cefpodoxime (Sankyo, Tokyo, Japan), cefmetazole (Sankyo), cefotaxime (Nippon Hoechst Marion Roussel, Tokyo, Japan), and cefepime (Bristol-Myers Squibb, Tokyo, Japan) were used as representative cephems. Other β-lactam agents, including imipenem (Banyu Pharmaceutical, Tokyo, Japan) as well as chloramphenicol (Sankyo) and rifampin (Sigma Chemical, St. Louis, Mo.), were also used. Clavulanic acid (SmithKline Beecham Pharmaceuticals, Tokyo, Japan) was used as a β-lactamase inhibitor.

Drug susceptibility assay. The susceptibility profiles were determined by the agar dilution method with sensitivity disk agar (Eiken Chemical, Tokyo, Japan) according to the guidelines of NCCLS (29).

Transconjugation. Conjugation was carried out by a broth method as described previously (20). Exponential-phase Luria broth cultures of donor strain KU6400 and recipient strain ML4947 were mixed at a ratio of 1:10 (by volume). This mating mixture was incubated for 2 h at 35°C. The transconjugants were selected on sensitivity disk agar containing rifampin at 64 μg/ml and cefpodoxime at 4 μg/ml.

Assay for β-lactamase. Crude extraction of AmpC β-lactamase was performed as described previously (32). Cells were harvested by centrifugation (1,700 × *g*, 10 min), resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.0), and sonicated. After centrifugation at 14,000 × *g* for 10 min at 4°C, β-lactamase activity was measured by determination of the protein content of the extract, and the protein contents of the cultures were compared. β-Lactamase activity was determined by spectrophotometry (UV2000; Shimadzu, Tokyo, Japan) at 30°C in 50 mM phosphate buffer (pH 7.0). Protein content was determined by a protein assay (Bio-Rad Laboratories, Hercules, Calif.) (10). One unit of β-lactamase activity was defined as the amount of β-lactamase that hydrolyzed 1 μmol of cephalothin in 1 min at 30°C. Cefoxitin (10 μg/ml) was used as the inducer. Induction was allowed to proceed for 60 min (4).

Cloning of the *ampC* and *ampR* genes. DNA extraction, restriction enzyme digestion, recombinant DNA manipulation, and transformations of plasmid DNA were performed as described by Sambrook et al. (40). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Tokyo, Japan), respectively. Plasmid pKU601 DNA was isolated from *E. coli* ML4947(pKU601) by the alkaline lysis method (8). The DNA was digested with BamHI and BglII and ligated into the BamHI site of pHSG398. The recombinant plasmid was designated pKU611 and was introduced into *E. coli* ML4947 by electroporation with a gene pulse controller unit (Bio-Rad

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics[a]
Bacterial strains	
E. coli	
ML4947	F⁻ *galK2 galT22 hsdR hsdM lacY1 metB1 relA supE*44 Rif^r (AmpD wild type)
ML4953	F⁻ *galK2 galT22 hsdR hsdM lacY1 metB1 relA supE*44 Rif^r *ampD9* (AmpD mutant)
KU6400	Clinical isolate carrying pKU601
C. freundii GC3	Clinical isolate from Japan which produces AmpC β-lactamase
Plasmids	
pKU601	Conjugative plasmid encoding CFE-1 isolated from KU6400
pKU611	14-kb fragment containing bla_{CFE-1} gene and *ampR* gene from pKU601 cloned into pHSG398
pKU612	Recombinant plasmid containing *ampC* gene of pKU611 in pHSG398
pHSG398	Cloning vector, purchased from Nippon Gene (Tokyo); Chl^r

[a] Characteristics include designation of markers, source, or derivation. Rif, rifampin; Chl, chloramphenicol.

TABLE 2. MICs of selected antibiotics for *E. coli* strains

Strain	MIC (μg/ml)[a]									
	PIP	CPD	CEF	CTX	CTX-CLA[b]	CAZ	CMZ	ATM	FEP	IPM
KU6400	>256	>256	>256	64	64	64	64	8	0.25	0.25
ML4947(pKU601)	>256	>256	>256	256	256	>256	256	64	1	0.5
ML4953(pKU601)	>256	>256	>256	256	256	>256	256	64	1	1
ML4947(pKU611)	64	>256	>256	16	16	8	16	2	0.25	0.25
ML4947(pKU612)	8	32	256	4	2	4	2	0.5	<0.063	0.25
ML4947	2	0.5	8	<0.063	<0.063	0.25	0.5	<0.063	<0.063	<0.063

[a] Antibiotics: PIP, piperacillin; CEF, cephalothin; CPD, cefpodoxime; CTX, cefotaxime; CLA, clavulanic acid; CAZ, ceftazidime; CMZ, cefmetazole; ATM, aztreonam; FEP, cefepime; IPM, imipenem.
[b] MICs were determined in the presence of clavulanic acid (5 μg/ml).

Laboratories). Transformants (containing *ampC* and *ampR*) were selected on the basis of resistance to cefpodoxime (4 μg/ml) and chloramphenicol (25 μg/ml) after overnight incubation at 37°C and were further characterized by analysis of their antibiotic susceptibility patterns. The size of the insert in the plasmid was estimated by restriction enzyme digestion and electrophoresis in 1.2% agarose gels.

To construct a plasmid containing only the *ampC* gene, plasmid pKU612, pKU611 was digested with SacI and ligated into the SacI site of pHSG398. The resulting plasmid was used to transform *E. coli* ML4947, and the plasmid from which the fragment containing the *ampR* sequence was deleted was identified from the plasmid DNA size and by DNA sequencing.

DNA sequencing and sequence comparisons. Sequencing of both strands of DNA was carried out as described by Sanger et al. (41) with a BigDye terminator cycle sequencing kit and an ABI 310 DNA sequencer (Applied Biosystems, Foster City, Calif.). Sequence analysis and comparison with other known sequences were performed with the BLAST and FAST programs at the National Center for Biotechnology Information.

Nucleotide sequence accession number. The nucleotide sequence data presented in this report appear in the DDBJ, EMBL, and GenBank nucleotide databases under accession number AB107899.

RESULTS

Susceptibilities to antibiotics. The MICs of selected β-lactam antibiotics for clinical isolate *E. coli* KU6400 and the transconjugants that acquired pKU601 by conjugation at a frequency of 10^{-5} are given in Table 2. KU6400 was highly resistant to piperacillin, cefotaxime, the combination of cefotaxime and clavulanic acid, aztreonam, and all cephalosporins except cefepime. The MICs of a variety of different cephalosporins were increased for the transconjugants.

Cloning of the *ampC* and *ampR* genes and gene expression in the *E. coli* recipient. We selected seven *E. coli* transformants resistant to β-lactams in a manner similar to that of *E. coli* ML4947 that acquired pKU601 by conjugation (Table 1). These transformants were resistant to cefpodoxime, cefotaxime, and chloramphenicol.

All transformants were found to produce an AmpC β-lactamase; they harbored a recombinant plasmid (pKU611) with an insert of 14 kb from pKU601. *E. coli* isolates harboring pKU611 showed a resistance profile similar to that of pKU601 (Table 2). Addition of clavulanic acid did not modify the resistance pattern in any transformant.

Characterization of the bla_{CFE-1} gene. Both strands of the entire 14-kb insert from recombinant plasmid pKU611 were sequenced. Analysis of this insert for coding regions revealed two open reading frames (ORFs) (Fig. 2). The first consisted of 1,137 bp encoding a putative protein of 378 amino acids (Fig. 3). This ORF had an ATG start codon at position 1008 and a stop codon at position 2151. Database searches with this ORF identified similarities with several chromosome- and plasmid-encoded AmpC β-lactamases, particularly the chromosome-encoded AmpC β-lactamase of *C. freundii* GC3 (99.8% sequence identity) (17). The deduced amino acid sequence carried catalytic residues S-X-X-K, with the initial serine at position 64 (which is typical of AmpC β-lactamases); the motif Y-S-N at position 150; and the K-T-G motif at position 315.

The second ORF, which contained 876 nucleotides was transcribed in the opposite orientation and was located in the 5′ direction from the *ampC* structural gene. It began with an ATG start codon at nucleotide 876 and had a stop codon at nucleotide 3. By analogy with the *ampC* and *ampR* genes of the AmpC β-lactamase, this ORF may correspond to the regulatory gene *ampR*. A sequence corresponding to transcriptional regulators of the LysR family, particularly the AmpR proteins of the family *Enterobacteriaceae*, was deduced. The DNA sequence of the corresponding gene from *C. freundii* GC3 was 99.0% identical to the sequence of this ORF. The deduced protein sequence showed only one difference, at position 135 (Ala for Asp), compared to the sequence of *C. freundii* GC3.

The 131-bp region between the *ampR* and *ampC* start codons contained overlapping putative promoters. This region was 97% identical to the corresponding region of *C. freundii* GC3.

Sequences surrounding *ampR* and *ampC* regions. In addition to identifying the sequences in the regions surrounding the *ampR* and *ampC* sequences, sequence analysis indicated that the *frdABCD* operon of *C. freundii* (7) was located upstream from the *ampC* gene; and a part of the ORF contained the sequence for the outer membrane lipoprotein encoded by the *blc* gene of *C. freundii* (GenBank accession nos. D85910 and U21727), which was located immediately downstream of the *ampC* gene (Fig. 4). Furthermore, two IS*26* elements were observed to surround the *ampR* and *ampC* genes and were directed in the same orientation, forming an IS*26* composite transposon. One was inserted in the *frdA* gene (as detected by PCR), and another was inserted in the *blc* gene.

β-Lactamase activities. The β-lactamase activities encoded by the plasmids are shown in Table 3. *E. coli* ML4947 (pKU601) and ML4953(pKU601) produced large amounts of β-lactamase (10.9 and 14.4 U/mg of protein, respectively). When *E. coli* ML4953 (AmpD mutant) was used as the host, the β-lactamase activities encoded by pKU601 were slightly higher than those detected when *E. coli* ML4947 (AmpD wild type) was used as the host. The β-lactamase activities of these strains in the presence of cefoxitin were slightly increased com-

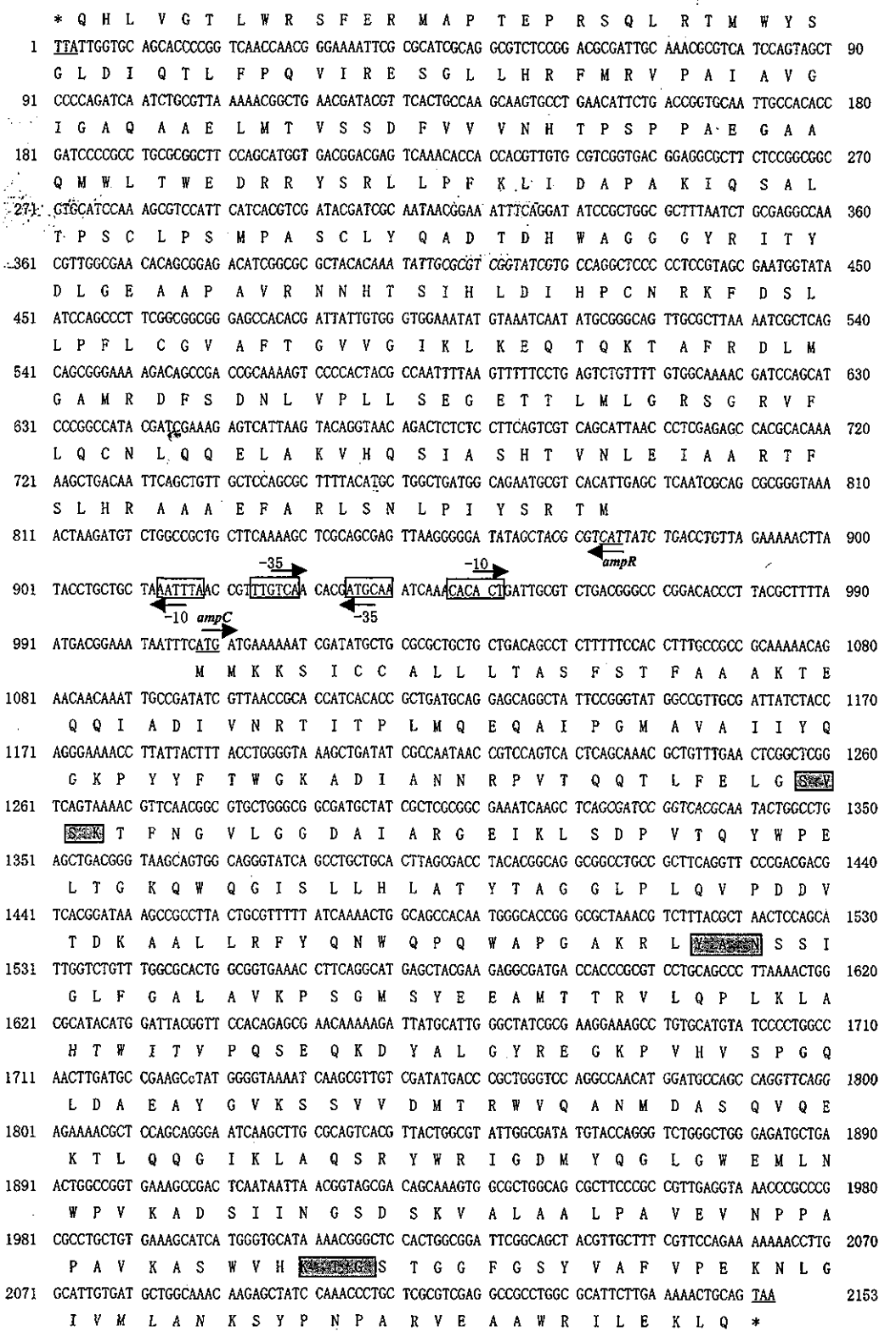

FIG. 2. Nucleotide sequence of the 2,153-bp fragment of pKU611 containing the *ampC*- and *ampR*-coding regions. Deduced amino acid sequences are designated in single-letter code. Putative promoter sequences are represented by the −35 and −10 regions (boxed). The start and stop codons of these genes are underlined. Additionally, conserved residues among class C β-lactamases are shown in shaded boxes.

```
CFE-1          MMKKSICCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYY 60
AmpC Cit GC3   ····························································
AmpC Cit OS60  ····························································
CMY-2          ·····L·····································V·····
AmpC Ent P99   ··R··L·····GI·C·AL·TPVS·K·L·EV·AN·····KA·SV·····V·····H·

CFE-1          FTWGKADIANNRPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTQYWPELTGKQ 120
AmpC Cit GC3   ····························································
AmpC Cit OS60  ··········H·····················R···········K·····
CMY-2          ··········H·····························K·····
AmpC Ent P99   Y·F·····A·K···P·····I····T···········S·D·A··R···Q·····

CFE-1          WQGISLLHLATYTAGGLPLQVPDDVTDKAALLRFYQNWQPQWAPGAKRLYANSSIGLFGA 180
AmpC Cit GC3   ····························································
AmpC Cit OS60  ·R·····I·G·····E·····T·····
CMY-2          ····R·····I····R·····H·····T·····
AmpC Ent P99   ····RM·D·····E···N·S·····K··TT·····A·····

CFE-1          LAVKPSGMSYEEAMTTRVLQPLKLAHTWITVPQSEQKDYALGYREGKPVHVSPGQLDAEA 240
AmpC Cit GC3   ·····W·····
AmpC Cit OS60  ····S·····R·····N··W··L·····
CMY-2          ·····R·····N·····W·····
AmpC Ent P99   ·····P··Q·····K····D····N··KA·EAH··W···D··A·R····M···Q·

CFE-1          YGVKSSVVDMTRWVQANMDASQVQEKTLQQGIKLAQSRYWRIGDMYQGLGWEMLNWPVKA 300
AmpC Cit GC3   ····························L··
AmpC Cit OS60  ·····I··A·····H·····E·····L··
CMY-2          ·····I··A·····H·····A·····L··
AmpC Ent P99   ····TN·Q··AN··M···APEN·ADAS·K···A·····S·····E·

CFE-1          DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLA 360
AmpC Cit GC3   ····························································
AmpC Cit OS60  ····························································
CMY-2          ····························································
AmpC Ent P99   NTVVE·····P··VA·····P·····I···QI·····

CFE-1          NKSYPNPARVEAAWRILEKLQ.                                        381
AmpC Cit GC3   ····················.
AmpC Cit OS60  ····················.
CMY-2          ·····V·····.
AmpC Ent P99   ·T·····YH···A··.
```

FIG. 3. Derived amino acid sequence of the CFE-1 β-lactamase compared to the sequences of other selected class C β-lactamases. The alignments of the deduced amino acid sequence of the CFE-1 β-lactamase with the AmpC β-lactamases of *C. freundii* GC3 (AmpC Cit GC3), *C. freundii* OS60 (AmpC Cit OS60), and *E. cloacae* P99 (AmpC Ent P99) and with the CMY-2 β-lactamase are shown. Dots indicate identical amino acids at that residue.

pared with the basal level, but the increases were not significantly different. This suggests that the bla_{CFE-1} gene may produce the enzyme constitutively.

The activity of CFE-1 was not inhibited by clavulanic acid, a characteristic confirming the close resemblance of CFE-1 to the AmpC β-lactamase, as mentioned in the description of the nucleotide sequence.

pKU611, which encoded the *ampR* and *ampC* genes, and recombinant plasmid pKU612, which encoded only the *ampC* gene (i.e., it lacked the *ampR* gene), were introduced into *E. coli* ML4947; and the β-lactamase activities of each construct were analyzed (Table 3). The specific enzyme activity of *E. coli* ML4947(pKU611) was 1.4 U/mg of protein, while that of *E. coli* ML4947(pKU612) was 0.2 U/mg of protein, a markedly lower level of expression.

DISCUSSION

In addition to previous reports concerning the MOX-1 and CMY-9 plasmid-encoded AmpC β-lactamases, which were detected in an *E. coli* clinical isolate in Japan (12, 19), in the present study we characterized a novel AmpC β-lactamase gene, bla_{CFE-1}, in a Japanese *E. coli* clinical isolate. This is the first report in East Asia of a plasmid-encoded AmpC β-lactamase, CFE-1, carrying an *ampR* gene derived from the *C. freundii* chromosome.

Our findings depict the organization of sequences surrounding the *ampR-ampC* region, including bla_{CFE-1}, which is seen in various enterobacterial species. Most plasmid-encoded AmpC β-lactamases, like CMY-2, CMY-4, and LAT-1, lack the *ampR* gene. *Citrobacter* spp. and *Enterobacter* spp. possess *ampR* and *ampC* genes, the fumarate operon *frdABCD* immediately

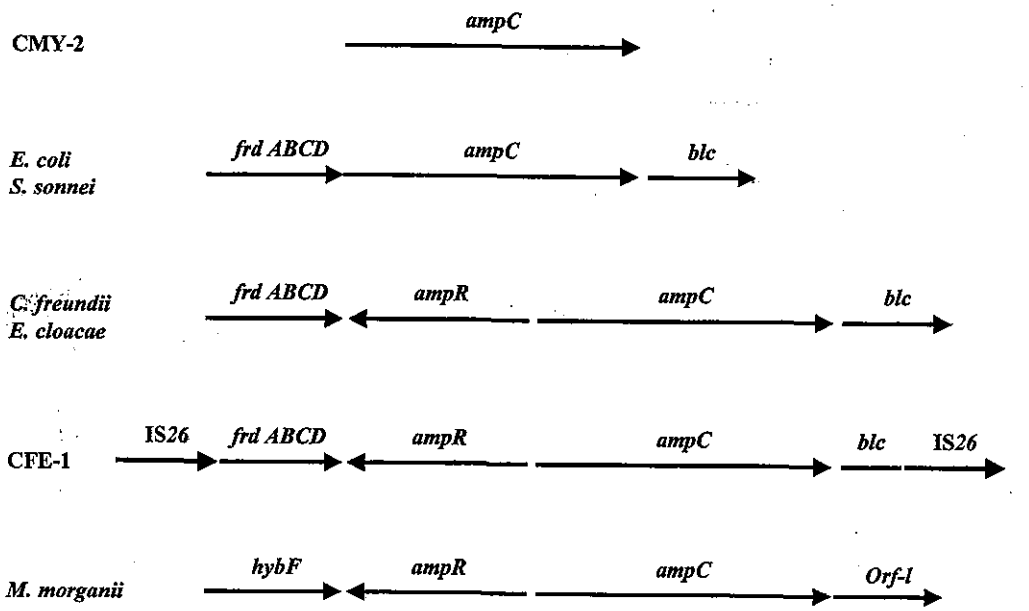

FIG. 4. Organization of the sequences surrounding *ampC* in various enterobacterial species. The positions of the fumarate operon (*frdABCD*), *blc*, *hybF*, *orf-1*, *IS26*, *ampC*, and *ampR* genes are shown, with directions indicated by arrows.

downstream of the *ampR* gene, and also outer membrane lipoprotein *blc* immediately downstream of the *ampC* gene (7, 9). In contrast, *M. morganii* possesses the *ampR* and *ampC* genes but not the fumarate operon (36); *hybF* is substituted for the fumarate operon upstream from the *ampC* gene (Fig. 4).

Analysis of the bla_{CFE-1} gene revealed that it has a very close relationship to the chromosomal gene that encodes the AmpC β-lactamase in *C. freundii* (1) (Table 4). The amino acid sequence of CFE-1 showed 99.5% identity with that of *C. freundii* GC3 isolated from clinical specimens in Japan (17), differing only at position 221 (Leu for Trp) and position 298 (Val for Leu) (Fig. 3). This identity was greater than that with *C. freundii* OS60 (95.0%) (26). The amino acid sequence of AmpR showed 99.0% identity with that of *C. freundii* GC3, differing only at position 135 (Ala for Asp). This similarity strongly suggests that the bla_{CFE-1} gene is derived from the chromosomal *ampC* gene of *C. freundii* GC3 (1). This hypothesis is supported by the finding that bla_{CFE-1} has both the *ampR* gene and the *ampC* gene, as well as the *frdABCD* gene operon and the *blc* gene, which were found to surround the *ampR* and *ampC* genes, as in *C. freundii*.

TABLE 3. β-Lactamase activities of *E. coli* strains

Strain	Relative β-lactamase activity (U/mg of protein)[a]	
	Noninduced	Induced[b]
KU6400	7.7	7.9
ML4947(pKU601)	10.9	11.1
ML4953(pKU601)	14.4	16.3
ML4947(pKU611)	1.4	1.7
ML4947(pKU612)	0.2	ND[c]

[a] β-Lactamase activities are the geometric mean determinations for three independent cultures. The standard deviations were within 10%.
[b] Cefoxitin (10 μg/ml); was used as the inducer.
[c] ND, not done.

In addition, two IS*26* elements were detected on plasmid pKU601; one was located immediately upstream from the *frdA* gene, while the other was located immediately downstream of the *ampC* gene and was inserted in the *blc* gene (Fig. 4). These were directed in the same orientation as that seen for the IS*26* composite transposon (25). Some plasmid-encoded β-lactamase genes form part of transposons frequently flanked by insertion sequence elements, such as IS*26* (SHV-2a and ACC-1) (22, 28) and IS*Ecp1* (CTX-M5) (39). These results present direct evidence that the bla_{CFE-1} gene translocated to a plasmid from the chromosome of *C. freundii* strain GC3 by using the IS*26* function(s). Further studies are continuing to determine whether the bla_{CFE-1} gene is capable of translocation.

As shown in Tables 2 and 3, an *E. coli* strain harboring pKU601 encoding bla_{CFE-1} expressed β-lactamase constitutively in the presence or absence of a β-lactam inducer and in the AmpD wild type (ML4947) or AmpD mutant (ML4953). *E. coli* ML4947(pKU612), which lacks the *ampR* gene, showed a decrease in β-lactamase activity compared with that of *E. coli* ML4947(pKU611); nevertheless, the AmpC β-lactamases of *C. freundii*, *E. cloacae*, and *M. morganii* with an *ampR* deletion showed increased levels of β-lactamase expression. This result

TABLE 4. Identity of the CFE-1 amino acid sequence to those of other AmpC β-lactamases

β-Lactamase	% Identity with:				
	CFE-1	GC3	OS60	CMY-2	P99
CFE-1	100	99.5	95.0	95.3	74.5
C. freundii GC3 AmpC		100	95.5	95.8	74.5
C. freundii OS60 AmpC			100	95.8	73.2
CMY-2				100	75.1
E. cloacae P99 AmpC					100

indicates that AmpR of pKU601 seems to function as regulator of the constitutive expression of bla_{CFE-1}. Bartowsky and Normark (3, 4) have reported that the activation of *ampC* transcription in *C. freundii* is dependent on the conversion of AmpR into a transcriptional activator. The AmpR mutants of *C. freundii*, which have Glu instead of Gly at position 102 or Tyl instead of Asp at position 135, express β-lactamase at high levels. Increased levels of β-lactamase expression have been reported (24) when the Arg-86 and Asp-135 mutations are present in *E. cloacae* AmpR. In the *ampR* gene of pKU601, the amino acid at position 135 was Ala, whereas it was Asp in the wild type. These results may indicate that overexpression of β-lactamase is dependent on mutation of the *ampR* gene.

In summary, *E. coli* plasmid pKU601 was characterized as harboring a novel plasmid-encoded AmpC β-lactamase, CFE-1, with an *ampR* gene. The high level of constitutive CFE-1 expression in *E. coli* is presumably caused by the mutation in the *ampR* gene, in which the Asp at position 135 is changed to Ala. These results indicate the dissemination of a resistance gene to different enterobacterial species through mobilization of a plasmid and transposable event-mediated events.

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REFERENCES

1. **Barlow, M., and B. G. Hall.** 2002. Origin and evolution of the AmpC β-lactamases of *Citrobacter freundii*. Antimicrob. Agents Chemother. **46**:1190–1198.
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. **Bartowsky, E., and S. Normark.** 1991. Purification and mutant analysis of *Citrobacter freundii* AmpR, the regulator for chromosomal AmpC β-lactamase. Mol. Microbiol. **5**:1715–1725.
4. **Bartowsky, E., and S. Normark.** 1993. Interactions of wild-type and mutant AmpR of *Citrobacter freundii* with target DNA. Mol. Microbiol. **10**:555–565.
5. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, and H. Giamarellou.** 1996. Characterization of the plasmidic β-lactamase CMY-2, which is responsible for cephamycin resistance. Antimicrob. Agents Chemother. **40**:221–224.
6. **Bennett, P. M., and I. Chopra.** 1993. Molecular basis of β-lactamase induction in bacteria. Antimicrob. Agents Chemother. **37**:153–158.
7. **Bergstrom, S., F. P. Lindberg, O. Olsson, and S. Normark.** 1983. Comparison of the overlapping *frd* and *ampC* operons of *Escherichia coli* with the corresponding DNA sequences in other gram-negative bacteria. J. Bacteriol. **155**:1297–1305.
8. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
9. **Bishop, R. E., S. S. Penfold, L. S. Frost, J. V. Holtje, and J. H. Weiner.** 1995. Stationary phase expression of a novel *Escherichia coli* outer membrane lipoprotein and its relationship with mammalian apolipoprotein D. Implications for the origin of lipocalins. J. Biol. Chem. **270**:23097–23103.
10. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
11. **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 foss of an outer membrane protein. Antimicrob. Agents Chemother. **41**:563–569.
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. **Fortineau, N., L. Poirel, and P. Nordmann.** 2001. Plasmid-mediated and inducible cephalosporinase DHA-2 from *Klebsiella pneumoniae*. J. Antimicrob. Chemother. **47**:207–210.
14. **Gaillo, O., C. Clement, M. Simonet, and A. Philippon.** 1997. Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. J. Antimicrob. Chemother. **39**:85–87.
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. **Hanson, N. D., and C. C. Sanders.** 1999. Regulation of inducible AmpC beta-lactamase expression among *Enterobactericeae*. Curr. Pharm. Des. **5**:881–894.
17. **Haruta, S., M. Nukaga, and T. Sawai.** 2001. Characterization of an extended-spectrum class C β-lactamase of *Citrobacter freundii*. Microbiol. Immunol. **45**:277–283.
18. **Honore, N., M. H. Nicolas, and S. T. Cole.** 1989. Regulation of enterobacterial cephalosporinase production: the role of a membrane-bound sensory transducer. Mol. Microbiol. **3**:1121–1130.
19. **Horii, T., Y. Arakawa, M. Ohta, T. Sugiyama, R. Wacharotayankun, H. Ito, and N. Kato.** 1994. Characterization of a plasmid-borne and constitutively expressed bla_{MOX-1} gene encoding AmpC-type β-lactamase. Gene **139**:93–98.
20. **Inoue, M., J. Itoh, and S. Mitsuhashi.** 1983. pMS76, a plasmid capable of amplification by treatment with chloramphenicol. Plasmid **9**:86–97.
21. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13**:4684–4694.
22. **Kim, J., H. S. Shin, S. Y. Seol, and D. T. Cho.** 2002. Relationship between bla_{SHV-12} and bla_{SHV-2a} in Korea. J. Antimicrob. Chemother. **49**:261–267.
23. **Korfmann, G., and C. C. Sanders.** 1989. *ampG* is essential for high-level expression of AmpC β-lactamase in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **33**:1946–1951.
24. **Kuga, A., R. Okamoto, and M. Inoue.** 2000. *ampR* gene mutations that greatly increase class C β-lactamase activity in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **44**:561–567.
25. **Lee, K. Y., J. D. Hopkins, and M. Syvanen.** 1990. Direct involvement of IS*26* in an antibiotic resistance operon. J. Bacteriol. **172**:3229–3236.
26. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii ampC* β-lactamase induction. Proc. Natl. Acad. Sci. USA **82**:4620–4624.
27. **Maejima, T., Y. Ohya, S. Mitsuhashi, and M. Inoue.** 1987. Cloning and expression of the gene(s) for chromosome-mediated β-lactamase production of *Proteus vulgaris* in *Escherichia coli*. Plasmid **18**:120–126.
28. **Nadjar, D., M. Rouveau, C. Verdet, L. Donay, J. Herrmann, P. H. 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.
29. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., vol. 17, no. 2. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
30. **Nordmann, P.** 1998. Trends in β-lactam resistance among Enterobacteriaceae. Clin. Infect. Dis. **27**(Suppl. 1):S100–S106.
31. **Normark, S.** 1995. β-Lactamase induction in gram-negative bacteria is intimately linked to peptidoglycan recycling. Microb. Drug Resist. **1**:111–114.
32. **Okamoto, R., T. Okubo, and M. Inoue.** 1996. Detection of genes regulating β-lactamase production in *Enterococcus faecalis* and *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40**:2550–2554.
33. **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.
34. **Perez-Perez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40**:2153–2162.
35. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46**:1–11.
36. **Poirel, L., M. Guibert, D. Girlich, T. Naas, and P. Nordmann.** 1999. Cloning, sequence analyses, expression, and distribution of *ampC-ampR* from *Morganella morganii* clinical isolates. Antimicrob. Agents Chemother. **43**:769–776.
37. **Reisbig, M. D., and N. D. Hanson.** 2002. The ACT-1 plasmid-encoded AmpC β-lactamase is inducible: detection in a complex β-lactamase background. J. Antimicrob. Chemother. **49**:557–560.
38. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.
39. **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.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
42. **Tzouvelekis, L. S., E. Tzelepi, A. F. Mentis, and A. Tsakaris.** 1993. Identification of a novel plasmid-mediated β-lactamase with chromosomal cephalosporinase characteristics from *Klebsiella pneumoniae*. J. Antimicrob. Chemother. 31:645–654.
43. **Verdet, C., G. Arlet, S. Ben Redjeb, A. Ben Hassen, P. H. Lagrange, and A. Philippon.** 1998. Characterisation of CMY-4, an AmpC-type plasmid-mediated β-lactamase in a Tunisian clinical isolate of *Proteus mirabilis*. FEMS Microbiol. Lett. **169**:235–240.

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Characterization of *ermB* Gene Transposition by Tn*1545* and Tn*917* in Macrolide-Resistant *Streptococcus pneumoniae* Isolates

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In *Streptococcus pneumoniae*, the *ermB* gene is carried by transposons, such as Tn*917* and Tn*1545*. This study investigated the relationship between macrolide resistance and the presence of the *ermB* gene on Tn*917* or Tn*1545* in 84 Japanese pneumococcal isolates. Macrolide-resistant strains were classified into two groups as follows. Group 1 (19 strains) showed a tendency to high resistance to erythromycin (MIC at which 50% of isolates are inhibited, 4 mg/liter; MIC at which 90% of isolates are inhibited [MIC_{90}], 128 mg/liter) but susceptibility to rokitamycin (MIC_{90}, 1 mg/liter), with the *ermB* gene located on Tn*1545*. Group 2 (65 strains) showed a tendency to high resistance to both antibiotics (MIC_{90}s for both erythromycin and rokitamycin, >128 mg/liter), with the *ermB* gene located on Tn*917*. There were no strains with constitutive macrolide resistance in either group. All of the strains in group 2 had a deletion in the promoter region of *ermB* and an insertion of the TAAA motif in the leader peptide. The results of pulsed-field gel electrophoresis and serogrouping showed that Tn*1545* spread clonally while Tn*917* spread both horizontally and clonally. In conclusion, in Japanese macrolide-resistant *S. pneumoniae* isolates, the *ermB* gene is carried and spread primarily by Tn*917*.

Streptococcus pneumoniae is still a major cause of respiratory tract infections, sinusitis, and acute otitis media. Antimicrobial resistance of *S. pneumoniae* has spread all over the world, and an increase in macrolide resistance has also been reported (1, 5, 7). In Japan, the macrolide resistance of *S. pneumoniae* has increased dramatically over the past decade and has reached a rate of 80% in clinical isolates, although there are some regional variations.

In *S. pneumoniae*, macrolide resistance is commonly caused by two major mechanisms: the efflux pump and target modification. The efflux pump, encoded by *mefA*, is associated only with resistance to 14- and 15-membered macrolides (19). On the other hand, target modification due to methylase, encoded by *ermB*, confers macrolide-lincosamide-streptogramin B (MLS_B) resistance (10). Among Japanese clinical isolates of *S. pneumoniae*, 42.7, 52.7, and 3.3% of strains have *mefA*, *ermB*, or both genes, respectively (6).

It has been reported that two transposons, Tn*1545* (22) and Tn*917* (17), carry the *ermB* gene in *S. pneumoniae*. Tn*1545* is almost identical to pAM77 from *Streptococcus sanguinis* (98% identity) and also mediates resistance to tetracycline via *tetM* (12) and resistance to kanamycin via *aphA-3* (4). In contrast, Tn*917* has no resistance genes for tetracycline or kanamycin and was identified on the nonconjugative multiple-resistance plasmid pAD2 in *Enterococcus faecalis* DS16 (17).

In this study, we investigated macrolide resistance among *S. pneumoniae* isolates in Japan with respect to (i) the relationship between transposon Tn*1545* or Tn*917* and resistance to macrolides, (ii) the question of whether macrolide resistance is inducible or constitutive, and (i) regional differences in the prevalence of the transposons in Japan.

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MATERIALS AND METHODS

Bacterial strains. Eighty-four macrolide-resistant *S. pneumoniae* strains were used in this study. These strains were initially selected by resistance to erythromycin and clindamycin (13). They were isolated between 1998 and 2003, at four hospitals and one laboratory in Japan, from the sputa of patients with lower respiratory tract infections and from the nasopharyngeal secretions or rhinorrhea of patients who had sinusitis with or without acute otitis media. The strains were preserved at our laboratory. All isolates were identified by their sensitivity to optochin and by the bile solubility test (16), as well as by PCR amplification of the *lytA* gene (8).

Antimicrobial agents. Reference powders of known potency of the following antimicrobial agents were used: penicillin G (Meiji Seika Kaisha, Ltd., Tokyo, Japan), erythromycin (Shionogi Pharmaceutical, Osaka, Japan), azithromycin (Pfizer Laboratories, Gorton, Conn.), rokitamycin (Asahi Kasei, Tokyo, Japan), and clindamycin (Upjohn, Tokyo, Japan). All of these antimicrobial agents were kind gifts from the respective manufacturers. Telithromycin (Nippon Hoechst Marion Roussel, Tokyo, Japan) was also used.

Determination of MICs. MICs were tested by using the six antibiotics mentioned above at concentrations between 0.06 and 128 mg/liter. MICs were determined by the twofold agar dilution method using sensitivity test agar (Mueller-Hinton agar medium; Eiken Chemicals, Tokyo, Japan) with 8% Strepto Haemo supplement (SHS; Eiken Chemicals). Bacteria were cultured overnight at 35°C in sensitivity test broth (Eiken Chemicals) supplemented with 8% SHS, after which the culture was diluted to a final concentration of 5×10^7 CFU/ml with buffered saline containing gelatin. The bacterial suspensions were then plated with an inoculator (Sakuma Seisaku, Tokyo, Japan) at an inoculum size of 5×10^4 CFU/spot onto agar plates containing various concentrations of a test drug. The plates were incubated for 18 h at 35°C, and the MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

Detection of resistance genes and analysis of the regulatory regions of the *ermB* gene. The presence of macrolide resistance genes was investigated by PCR using a commercially available kit (Gene Amp PCR kit with AmpliTaq DNA polymerase; Takara, Kyoto, Japan) and a model PH2000 DNA thermal cycler (Perkin-Elmer Cetus Instruments, Emeryville, Calif.). The primer set for the internal region of *ermB* comprised 5′-GAAAAGGTACTCAACCAAATA-3′ and 5′-AGTAACGGTACTTAAATTGTTTAC-3′, while that for the internal region of *mefA* comprised 5′-AGTATCATTAATCACTAGTGC-3′ and 5′-TTC