ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2005, p. 2133–2136 Vol. 49, No. 5
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.5.2133–2136.2005

Substrate-Dependent Utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK Efflux Pump

Rungtip Chuanchuen,[2] Takeshi Murata,[3] Naomasa Gotoh,[3] and Herbert P. Schweizer[1]*

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado 80523[1]; Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Patumwan, Bangkok, 10330, Thailand[2]; and Department of Microbiology, Kyoto Pharmaceutical University, Yamashina, Kyoto, 6078414, Japan[3]

Received 13 October 2004/Returned for modification 4 January 2005/Accepted 27 January 2005

MexJK requires OprM for erythromycin efflux but not for triclosan efflux. Deletion of 15 OprM family outer membrane proteins (OMPs) revealed that only the TolC homolog OpmH functions with MexJK for triclosan efflux. This is the first report of natural utilization of multiple OMPs by a given resistance nodulation cell division transporter/membrane fusion protein pair.

Efflux pumps of the resistance nodulation cell division family (RND) are associated with the intrinsic or acquired multidrug resistance phenotype of gram-negative bacteria (for recent reviews, see references 17 and 22). The RND pumps, as typified by AcrAB-TolC of *Escherichia coli* (8), form a tripartite complex spanning the entire cell envelope. High-resolution crystal structures of the outer membrane protein (OMP) component TolC (16), the RND membrane transporter AcrB (20, 26), and the *Pseudomonas aeruginosa* AcrA homolog MexA (1, 11) revealed a mechanism by which RND pumps can achieve this (7). Our previous studies showed that the MexJK efflux system required OprM for erythromycin efflux but not for triclosan efflux, and we reasoned that either MexJ and MexK were sufficient for triclosan efflux or MexJ and MexK interacted with an OMP other than OprM to form a functional triclosan efflux pump.

We previously determined that OprJ, OprM, and OprN did not function with MexJK to assemble an active triclosan efflux pump (4), although the respective OMP functioned with MexAB-OprM, MexCD-OprJ, and MexEF-OprN in triclosan efflux (2, 4). Because we suspected that an OMP of the 18-member OprM family might associate with MexJK, we decided to individually delete the genes encoding the remaining 15 members of this family in strain PAO327 constitutively expressing MexJK but not OprJ, OprM, and OprN. Although a comprehensive PAO1 transposon library was recently published which contains insertions in all OprM family genes (14), we decided to generate unmarked deletion mutants to avoid possible undesired effects resulting from truncated OMPs. The respective promoterless coding sequences were PCR amplified from PAO1 genomic DNA using gene-specific primers (unpublished data). Plasmid-borne deletions were generated and used to construct unmarked deletions in the PAO327 chromosome, utilizing published methods (12) (plasmids and strains used in this study are listed in Table 1; a comprehensive list of intermediate plasmids and strains is available from the authors). *opmJ* was deleted using a modified PCR-targeted method (5) in which the target gene is first cloned into a plasmid, followed by λRED-mediated recombination of a PCR-generated mutated copy of the gene (10). Because PCR amplification of the *oprN* gene proved futile for unknown reasons, an *oprN*::IS*lacZ*/hah-Tc transposon insertion from strain 8802 (14) was transferred to PAO327 using a previously described transformation procedure (9). Using these methods, we succeeded in deleting 14 of the 15 genes encoding the OprM family of OMPs and insertionally inactivating *oprN*. The resulting strains, except PAO375, contain well-defined unmarked deletion alleles and have between 21 and 395 codons deleted from the respective coding sequences. For complementation, the promoterless inserts were directionally cloned into pVLT35 (6), in which the DNA inserts are under the transcriptional control of the *E. coli tac* promoter and the *lac* repressor.

MICs for triclosan (KIC Chemicals, Armonk, NY) and erythromycin (Sigma, St. Louis, MO) were used as a measure of MexJK efflux pump activity in this panel of defined OMP mutants and their complemented derivatives. MICs were determined by the twofold broth microdilution technique (2). An agar incorporation method was used to determine triclosan MICs of >128 μg/ml (3). Deletion of only one gene, *opmH*, disrupted MexJK-mediated triclosan efflux, and overexpression of OpmH restored MexJK function to wild-type levels (Table 2). Deletion of *opmH* and overexpression of OpmH resulted in a marginal (twofold) decrease or increase, respectively, in the MIC of erythromycin, indicating that MexJK-OpmH may also be able to mediate some erythromycin efflux. Deletion of any of the other OprM family OMP genes and complementation with the corresponding genes expressed from the *tac* promoter did not have any significant effects on triclosan and erythromycin MICs, indicating that none of these OMPs can be utilized by MexJK to catalyze efflux of these antimicrobials. Reverse transcription-PCR analysis indicated that all genes were highly expressed in isopropyl-β-D-thiogalactopyranoside-induced cells, verifying that lack of complementation was not due to lack of expression (data not shown).

* Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, Colorado State University, 1682 Campus Delivery, Fort Collins, CO 80523. Phone: (970) 491-3536. Fax: (970) 491-1815. E-mail: Herbert.Schweizer@colostate.edu.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or properties[a]	Source or reference
PAO1	wild type	13
PAO325	Δ(*mexAB-oprM*)[b] *nfxB* Δ(*mexCD-oprJ*) Δ(*mexJKL*) Δ(*mexXY*) OpmH$^+$	4
PAO327	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexXY*) *mexL* MexJ$^+$K$^+$	4
PAO346	PAO327 with Δ*opmD*302[c]	This study
PAO351	PAO327 with Δ*opmM*81	This study
PAO353	PAO327 with Δ*aprF*153	This study
PAO355	PAO327 with Δ*opmL*133	This study
PAO357	PAO327 with Δ*opmF*123	This study
PAO359	PAO327 with Δ*opmG*151	This study
PAO361	PAO327 with Δ*opmA*21	This study
PAO363	PAO327 with Δ*opmB*282	This study
PAO365	PAO327 with Δ*opmE*209	This study
PAO367	PAO327 with Δ*opmH*362	This study
PAO369	PAO327 with Δ*opmK*53	This study
PAO371	PAO327 with Δ*opmJ*184	This study
PAO373	PAO327 with Δ*opmJ*177	This study
PAO375	PAO327 with *opmN*::IS*lacZ*/hah-Tc	This study
PAO377	PAO327 with Δ*opmQ*395	This study
PAO386	PAO325 with Δ*opmH*362	This study
PAO397	PAO386 with Δ(*mexEF-oprN*)	This study
8802	Tet^r; PAO1 with *opmN*::IS*lacZ*/hah-Tc	14
KG4510	Δ(*nfxB-mexCD-oprJ*) *mexR* Δ*oprM* Δ(*mexXY*)	21
pBSP II KS(−)/SK(−)	Ap^r; broad-host-range cloning vectors	23
pCR2.1	Ap^r; TA cloning vector for PCR fragments	Novagen
pVLT35	Sp^r/Sm^r; broad-host-range cloning vector	6
pPS1150	Ap^r; pBSP II SK(−) with 6,945-bp NotI *mexJKL* fragment	4
pPS1266	Ap^r; pCR2.1 with 1,673-bp *opmH* PCR fragment	This study
pPS1313	Sp^r/Sm^r; pVLT35 with 1,664-bp PstI-HindIII *opmH* fragment from pPS1266	This study
pPS1319	Ap^r; pCR2.1 with 1,563-bp EcoRV-HindIII *oprM* PCR fragment	This study
pPS1369	Ap^r; pCR2.1 SpeI(blunt)+XhoI with 4,369-bp SgfI(blunt)-XhoI *mexJK* fragment from pPS1150	This study
pPS1370	Ap^r; pBSP II KS(−) carrying a 4,394 bp BamHI-XbaI *mexJK* fragment from pPS1369	This study
pPS1371	Ap^r; pPS1370 BamHI(blunt)+HindIII with 1,530-bp EcoRV-HindIII *oprM* fragment from pPS1319	This study
pPS1372	Ap^r; pPS1370 BamHI(blunt)+HindIII with 1,659-bp PstI(blunt)-HindIII *opmH* fragment from pPS1266	This study
pPS1373	Ap^r; pPS1371 HindIII(blunt)+ClaI with 1,665-bp *Pst*I(blunt)-ClaI *opmH* fragment from pPS1389	This study
pPS1389	Ap^r; pBSP II KS(−) with 1,664-bp PstI-HindIII *opmH* fragment from pPS1266	This study
pPS1424	Ap^r; pBSP II KS(−)XhoI(blunt)+HindIII with 1,659-bp PstI(blunt)-HindIII *opmH* fragment from pPS1266	This study
pPS1425	Ap^r; pBSP II KS(−)XhoI(blunt)+HindIII with 1,530-bp *Eco*RV-HindIII *oprM* fragment from pPS1319	This study

[a] Abbreviations: Ap^r, ampicillin resistance; *FRT*, Flp recombinase target; Gm^r, gentamycin resistance; Sm^r/Sp^r, streptomycin/spectinomycin resistance; Tet^r, tetracycline resistance.
[b] All deletions are unmarked but contain an *FRT* scar insertion; e.g., Δ(*mexAB-oprM*)::*FRT*.
[c] Allele numbers indicate the number of codons deleted from the respective genes.

If OpmH is indeed the OMP used by MexJK for triclosan efflux, then OpmH must either be constitutively expressed or be coexpressed with MexJK. Utilizing previously described procedures (21), rabbit anti-OpmH polyclonal antiserum was developed by immunization with three oligopeptides, VGDT RIAFDERPATVKRN (amino acids 77 to 94), LNQSEQSRE GQRRQV (amino acids 341 to 355), and AEQLQSKPRQQY (amino acids 471 to 482), based on the deduced OpmH primary sequence (24). Membrane preparation and immunoblot analyses were performed as previously described (21). The anti-OpmH polyclonal antiserum did not cross-react with OprM, OprJ, OprN, OpmD, OpmE, and OpmB in membrane preparations of strains expressing these proteins constitutively but did react with native and histidine-tagged OpmH expressed in *P. aeruginosa* KG4510 (data not shown). Immunoblot analysis of total membrane proteins from PAO1 grown in LB medium (19) revealed growth phase-independent constitutive OpmH expression (Fig. 1). Similar observations were made when transcription of *opmH* was analyzed in LB-grown cells with an *opmH'-lacZ* transcriptional fusion and real-time PCR (data not shown). In both approaches, subinhibitory triclosan concentrations (8 μg/ml) did not significantly affect *opmH* expression, i.e., triclosan did not induce *opmH* transcription.

To further assess the roles of OprM and OpmH in MexJK-mediated erythromycin and triclosan efflux, we constructed hybrid operons containing *mexJK* alone, *mexJK-oprM*, *mexJK-opmH*, and *mexJK-oprM-opmH*, where constitutive transcription of all genes originated from the *mexJK* promoter. Plasmids containing these hybrid operons were transformed into strain PAO397, which carries deletions for five major efflux systems

TABLE 2. Contribution of *oprM* and *opmH* to erythromycin and triclosan efflux

Strain/plasmid	OMP gene deletion/complementation	Efflux components expressed	MIC (μg/ml)[a]	
			Tri	Ery
PAO327[b]	Δ*oprJ* Δ*oprM*	MexJK, OpmH	128	16
PAO367	Δ*oprJ* Δ*oprM* Δ*opmH*362	MexJK	16	8
PAO367/pVLT35[c]	Δ*oprJ* Δ*oprM* Δ*opmH*362	MexJK	16	8
PAO367/pPS1313[c]	Δ*oprJ* Δ*oprM* Δ*opmH*362/*opmH*$^+$	MexJK, OpmH	128	16
PAO397/pBSP II-KS	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*	None	2	8
PAO397/pPS1370[d]	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*	MexJK	2	8
PAO397/pPS1371	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$	MexJK, OprM	2	32
PAO397/pPS1372	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*opmH*$^+$	MexJK, OpmH	>1,024	8
PAO397/pPS1373	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$ *opmH*$^+$	MexJK, OprM, OpmH	512	64
PAO397/pPS1424	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*opmH*$^+$	OpmH	8	8
PAO397/pPS1425	Δ*oprM* Δ*oprJ* Δ*oprN* Δ*opmH362*/*oprM*$^+$	OprM	2	8

[a] Erythromycin (Ery) MICs were determined using the broth microdilution method, and triclosan (Tri) MICs were determined using the agar incorporation method, except for PAO327 and the PAO367 series of strains, for which the microdilution method was used.

[b] The genotype of PAO327 is Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexXY*) *mexL* (MexJK constitutively expressed) and MexEF-OprN uninducible due to a *mexT* insertion (18).

[c] In pVLT35 and pPS1313, *opmH* transcription is driven from the *lacI* repressor-controlled P_{tac} promoter; media used for MIC determinations with cells harboring these two plasmids were therefore supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (Gold Biotechnology, St. Louis, MO).

[d] Hybrid *mexJK* (pPS1370), *mexJK-oprM* (pPS1371), *mexJK-opmH* (pPS1372), and *mexJK-oprM-opmH* (pPS1373) operons were assembled on pBSP II KS(−). pPS1371 contains *oprM*, 52 bp of its upstream region and 13 bp of its downstream region without transcriptional terminator. pPS1372 and pPS1373 contain *opmH*, 72 bp of its upstream region and 136 bp of its downstream region, including the transcriptional terminator. The recombinant plasmids were transformed into Δ(*mexAB-oprM*) Δ(*mexCD-oprJ*) Δ(*mexEF-oprN*) Δ(*mexLJK*) Δ(*mexXY*) Δ*opmH*362 strain PAO397, and efflux of the indicated antimicrobials was assessed by MIC determinations. On pPS1370–pPS1373, constitutive transcription of the cloned genes originates from the *mexJK* operon promoter (P_{JK}). pPS1424 and pPS1425 are based on pVLT35, and transcription of cloned genes is driven from the *lacI* repressor-controlled P_{lac} promoter; media used for MIC determinations with cells harboring these two plasmids were therefore supplemented with 1 mM IPTG.

(including *oprJ*, *oprM*, and *oprN*) and the Δ*opmH*362 mutation. MICs for erythromycin and tetracycline were determined as a measure of efflux of the respective antimicrobials (Table 2). Expression of all genes was verified by reverse transcription-PCR (data not shown). It is evident that MexJK requires OprM for erythromycin efflux and OpmH for triclosan efflux, since in the absence of OprM and OpmH neither erythromycin nor triclosan efflux is observed. As expected, simultaneous expression of OprM and OpmH led to efflux of both antimicrobials, although triclosan efflux was somewhat lower when compared to efflux by the MexJK-OpmH pump. This is perhaps due to competition of OpmH and OprM for formation of a functional MexJK-OpmH triclosan efflux pump. Erythromycin efflux was marginally (twofold) higher in the strain expressing both OprM and OpmH when compared to efflux observed in the MexJK-OprM-expressing strain. Expression of OprM alone had no effect, indicating that the observed triclosan and erythromycin efflux was not due to the mere presence of this OMP alone or its interaction with yet another efflux system. In contrast, overexpression of OpmH resulted in a significant (fourfold) increase in the MIC for triclosan, indicating that OprM may be able to interact with yet another efflux system to mediate triclosan efflux. Of note also is that the triclosan MICs for PAO367 (16 μg/ml) and PAO397 (2 μg/ml) are significantly different, but we do not yet understand the cause for this difference.

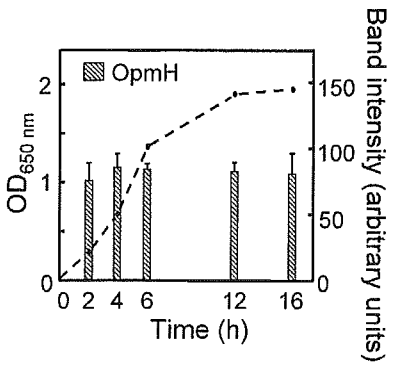

FIG. 1. Constitutive production of OpmH in *P. aeruginosa*. Cells of PAO1 were grown in LB medium, and aliquots were removed at the indicated time points after subculture. Total membranes (5 μg of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using anti-OpmH polyclonal antiserum. Relative expression levels of OpmH were determined by quantifying band intensities using the Scion Image program (Scion Corporation, Frederick, MD). Each bar represents the mean ± standard deviation of the intensity levels obtained in four experiments. The average optical density (650 nm) of the cultures used for sample preparation is overlaid.

The data presented here confirm that triclosan efflux in a strain expressing MexJK is mediated by a tripartite MexJK-OpmH efflux system. The constitutive expression and apparent absence of *opmH* cotranscribed efflux or secretion components confirm the previously published notion that the TolC homolog OpmH may play a more general role by interacting with more than one efflux system (15). However, the data presented here clearly indicate that these interactions may not be nonspecific, as previously suggested, but in some instances may rather depend on which substrate is being exported by the respective efflux system. How the OMP exerts its impact on substrate specificity of the pump remains unclear. One possibility is that the specific substrate export pathway, e.g., by opening of the exit pore, is formed only when all three proteins are properly assembled in the presence of the appropriate substrate. Another possibility is a substrate-induced assembly of the tripartite efflux pump, i.e., by recruitment of the appro-

priate OMP, similar to what has previously been observed with the HlyBD-TolC protein export system (25).

H.P.S. was supported by NIH grant AI051588, and N.G. was supported by grants from the Ministry of Education, Sports, Science and Technology and the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

1. **Akama, H., T. Matsuura, S. Kashiwagi, H. Yoneyama, S.-I. Narita, T. Tsukihara, A. Nakagawa, and T. Nakae.** 2004. Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. J. Biol. Chem. **279**:25939–25942.
2. **Chuanchuen, R., K. Beinlich, T. T. Hoang, A. Becher, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrob. Agents Chemother. **45**:428–432.
3. **Chuanchuen, R., R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2003. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely due to efflux. Am. J. Infect. Control **31**:124–127.
4. **Chuanchuen, R., C. T. Narasaki, and H. P. Schweizer.** 2002. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. J. Bacteriol. **184**:5036–5044.
5. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97**:6640–6645.
6. **De Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/P*trp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123**:17–24.
7. **Eswaran, J., E. Koronakis, M. K. Higgins, C. Hughes, and V. Koronakis.** 2004. Three's company: component structures bring a closer view of tripartite drug efflux pumps. Curr. Opin. Struct. Biol. **14**:741–747.
8. **Fralick, J. A.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178**:5803–5805.
9. **Gallagher, L. A., S. L. McKnight, M. S. Kuznetsova, E. C. Pesci, and C. Manoil.** 2002. Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. J. Bacteriol. **184**:6472–6480.
10. **Gust, B., G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA **100**:1541–1546.
11. **Higgins, M. K., E. Bokma, E. Koronakis, C. Hughes, and V. Koronakis.** 2004. Structure of the periplasmic component of a bacterial drug efflux pump. Proc. Natl. Acad. Sci. USA **101**:9994–9999.
12. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**:77–86.
13. **Holloway, B. W.** 1955. Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **13**:572–581.
14. **Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. H. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. K. Raymond, R. Levy, L. Chun-Rong, D. Guenthner, D. Bovee, M. V. Olson, and C. Manoil.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **100**:14339–14344.
15. **Jo, J., F. S. L. Brinkman, and R. E. W. Hancock.** 2003. Aminoglycoside efflux in *Pseudomonas aeruginosa*: involvement of novel outer membrane proteins. Antimicrob. Agents Chemother. **47**:1101–1111.
16. **Koronakis, V., A. Sharff, E. Koronakis, B. Luisi, and C. Hughes.** 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature **405**:914–919.
17. **Li, X. Z., and H. Nikaido.** 2004. Efflux-mediated drug resistance in bacteria. Drugs **64**:159–204.
18. **Maseda, H., K. Saito, A. Nakajima, and T. Nakae.** 2000. Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **192**:107–112.
19. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Murakami, S., R. Nakashima, E. Yamashita, and A. Yamaguchi.** 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature **419**:587–593.
21. **Murata, T., N. Gotoh, and T. Nishino.** 2002. Characterization of outer membrane efflux proteins OpmE, OpmD and OpmB of *Pseudomonas aeruginosa*: molecular cloning and development of specific antisera. FEMS Microbiol. Lett. **217**:57–63.
22. **Poole, K.** 2004. Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. **10**:12–26.
23. **Schweizer, H. P.** 2001. Vectors to express foreign genes and techniques to monitor gene expression in Pseudomonads. Curr. Opin. Biotechnol. **12**:439–445.
24. **Stover, C. K., X.-Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, D. Spencer, G. K.-S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa*, an opportunistic pathogen. Nature **406**:959–964.
25. **Thanabalu, T., E. Koronakis, C. Hughes, and V. Koronakis.** 1998. Substrate-induced assembly of a contiguous channel for protein export from *E. coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. EMBO J. **17**:6487–6496.
26. **Yu, E. W., G. McDermott, H. I. Zgurskaya, H. Nikaido, and D. E. Koshland, Jr.** 2003. Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. Science **300**:976–980.

Microbiol. Immunol., **49**(5), 443–446, 2005

Cooperation between Alteration of DNA Gyrase Genes and Over-Expression of MexB and MexX Confers High-Level Fluoroquinolone Resistance in *Pseudomonas aeruginosa* Strains Isolated from a Patient Who Received a Liver Transplant Followed by Treatment with Fluoroquinolones

Toshiyuki Niga[1], Hideaki Ito*[,1], Yoshihiro Oyamada[1], Jun-ichi Yamagishi[1], Manami Kadono[2], Takeshi Nishino[2], Naomasa Gotoh[2], and Matsuhisa Inoue[3]

[1]*Pharmacology & Microbiology Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Suita, Osaka 564–0053, Japan,* [2]*Department of Microbiology, Kyoto Pharmaceutical University, Kyoto, Kyoto 607–8414, Japan, and* [3]*Department of Microbiology, Kitasato University School of Medicine, Sagamihara, Kanagawa 228–8555, Japan*

Received December 29, 2004; in revised form, February 21, 2005. Accepted March 1, 2005

Abstract: **Clinical isolates of highly fluoroquinolone-resistant *Pseudomonas aeruginosa* had a mutation in either A or B subunit of DNA gyrase and over-expressed MexB and MexX, the efflux system proteins. Introduction of wild-type gyrase genes of *Escherichia coli* into the isolates made them as fluoroquinolone-susceptible as the moderately fluoroquinolone-resistant strains that only over-expressed efflux system proteins. These findings demonstrate that high fluoroquinolone-resistance in *P. aeruginosa* is attributed to cooperation between alteration in DNA gyrase genes and over-expression of efflux systems proteins.**

Key words: **Fluoroquinolone-resistance, *Pseudomonas aeruginosa*, DNA gyrase, Efflux systems**

Pseudomonas aeruginosa is a major human opportunistic pathogen characterized by intrinsic resistance to a variety of structurally unrelated antibacterial agents. Although fluoroquinolones (FQs) are the most useful oral agents in the treatment of *P. aeruginosa* infections, *P. aeruginosa* ability to quickly become resistant to FQs remains a major clinical problem. Two major mechanisms have been proposed for *P. aeruginosa* resistance to FQs. One is alteration of target enzymes, such as DNA gyrase (heterotetramer in the form of A_2B_2) and topoisomerase IV (heterotetramer in the form of C_2E_2), caused by mutations in the so-called quinolone resistance-determining region (QRDR) (3, 5, 14). The other is over-expression of proteins that construct different efflux systems, i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (4, 7, 10, 11). While these two major mechanisms have been examined independently in clinical isolates of *P. aeruginosa*, little is known of the functional cooperation between them.

In order to determine whether *P. aeruginosa* acquires high-level FQ resistance by both altering target enzymes and constructing efflux systems, eleven *P. aeruginosa* strains isolated from a patient who received a liver transplant followed by treatment with FQs were examined for mutations in QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes and expression of proteins constructing efflux systems. Complementation tests were carried out with wild-type *E. coli* gyrase genes, *gyrA* and *gyrB*.

Since *P. aeruginosa* strains were isolated on different dates and from different sources, we first examined, using pulsed-field gel electrophoresis (PFGE), whether they had the same genetic background. The eleven isolates had the same PFGE pattern (determined as described by Anthony et al. [2]) and were confirmed

**Address correspondence to* Dr. Hideaki Ito, Pharmacology & Microbiology Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Enoki 33–94, Suita, Osaka 564–0053, Japan. Fax: +81–6–6338–7656. E-mail: hideaki-ito@dainippon-pharm.co.jp

Abbreviations: CPFX, ciprofloxacin; FQ, fluoroquinolone; LVFX, levofloxacin; MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis; QRDR, quinolone resistance-determining region.

Table 1. Characteristics of GyrA and GyrB, and expression of MexB and MexX in clinical strains of *P. aeruginosa* isolated from a patient who received a liver transplant

Isolate	Date of isolation	Source	MIC (μg/ml)[a] of:		Mutation in QRDR of:		Expression[b] of:	
			LVFX	CPFX	GyrA	GyrB	MexB	MexX
K101	4/5/99	BAF[c]	0.5	0.125			+	−
K102	4/21/99	Blood	0.5	0.125			+	−
K103	5/14/99	Sputum	2	0.5			+	−
K104	5/17/99	Liver	1	0.25			+	−
K105	6/7/99	Sputum	16	2		E470D	+++	++
K106	6/11/99	Bile	4	0.5			++	+++
K107	7/15/99	Bile	32	16	T83I		+++	+++
K108	8/27/99	Sputum	32	16	T83I		+++	+++
K109	9/1/99	Feces	32	16	T83I		+++	+++
K110	9/24/99	Broncho	32	8	T83I		+++	+++
K111	9/24/99	Liver	4	1			++	++
PAO1[d]			0.5	0.06			+	−

[a] LVFX, levofloxacin; CPFX, ciprofloxacin.
[b] Semiquantitative values are expressed as a range from − (negative) to +++ (strongly positive).
[c] Bronchoalveolar lavage fluid.
[d] Reference strain.

clonal (data not shown).

Susceptibility of each isolate to levofloxacin (LVFX) and ciprofloxacin (CPFX) was examined using the agar dilution method as recommended by NCCLS (9). Generally, minimum inhibitory concentrations (MICs) of LVFX and CPFX against *P. aeruginosa* strains varied remarkably between isolates with higher values for strains isolated in the latter half of this study (Table 1). For isolates collected from the same source, the latest isolates were more resistant to LVFX and CPFX than those isolated earlier in the study. According to NCCLS standards, K105 isolate was resistant to LVFX (MIC≧8), and K107, K108, K109, and K110 isolates were resistant to both LVFX and CPFX (MIC≧4). K107, K108, and K109 isolates exhibited particularly strong resistance to LVFX and CPFX with MICs of 32 and 16 μg/ml, respectively. These MIC values were 64- to 128-fold higher than those for K101, the strain isolated initially.

Involvement of target enzyme alteration in FQ resistance was confirmed by DNA sequencing of QRDRs. QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR using KOD-Plus-DNA polymerase (TOYOBO, Japan) and the following primers: PAGYRAF1 (5′-GCCAAAGAAATTCTCCCGGT-3′) and PAGYRAR1 (5′-ATGTACTGCATCAGCTCATC-3′) for QRDR of *gyrA* gene, PAGYRBF1 (5′-GTCTCCTCCGAGGTGAAGA-3′) and PAGYRBR1 (5′-TCGATCAGCTCGGGCATCT-3′) for QRDR of *gyrB* gene, PAPARCF1 (5′-CTGCGCCAAAACACTTCTC-3′) and PAPARCR1 (5′-CGTGTTCGCACAATTCGG-3′) for QRDR of *parC* gene, and PAPAREF1 (5′-AGCGGATCGCCTTCGTCC-3′) and PAPARER1 (5′-TTCGTCGAGGGCGTAGTAG-3′) for QRDR of *parE* gene. PCR products were sequenced with an Applied Biosystems 3100 Sequencer. DNA sequencing of QRDRs revealed that the four isolates (K107, K108, K109, and K110), which showed high resistance to LVFX had a point mutation, i.e. ACC (Thr-83) to ATC (Ile) in the *gyrA* gene and that other isolates had no mutation in the QRDR of *gyrA* gene. In addition, none of the isolates had point mutation in the QRDR of *parC* gene. Although high resistance to LVFX is commonly believed to be associated with a point mutation in *gyrA* and/or *parC* genes, K105 isolate, which exhibited relatively high resistance to LVFX, had no mutation in these genes. Interestingly, the K105 isolate had a point mutation with GAG (Glu-470) to GAT (Asp) in the *gyrB* gene. On the other hand, no mutation was found in the QRDR of *parE* gene in all isolates. The changes in amino acid sequence found in this study, i.e. Thr-83 to Ile in *gyrA* and Glu-470 to Asp in *gyrB*, have already been reported by Akasaka et al. (1). However, the functionality of these changes, particularly that in the *gyrB* gene, has not been investigated.

To determine whether Glu-470 to Asp point mutation in *gyrB* gene contributes to FQ resistance, we investigated the involvement of four active efflux systems in FQs resistance. The amounts of MexB, MexX, MexC, and MexE proteins constructing MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN systems in the isolates were determined by immunoblot assay using rabbit polyclonal antisera specific for MexB, MexX, MexC, and MexE as described previously (6, 13). Production levels of MexB and MexX in the isolates that were resistant to LVFX, i.e. K105, K107,

Table 2. Changes in susceptibility to levofloxacin in isolates carrying wild-type *gyrA* or *gyrB* alleles

Isolate	Levofloxacin MIC (μg/ml) for isolates with:		
	No plasmid	pPAW207 (*gyrA*)	pPBW801 (*gyrB*)
K101	0.5	0.5	0.5
K102	0.5	0.5	0.5
K103	2	1	4
K104	1	1	2
K105	16	16	2
K106	4	2	4
K107	32	4	64
K108	32	4	64
K109	32	4	64
K110	32	4	64
K111	4	2	8
PAO1[a)]	0.5	0.25	0.25

[a)] Reference strain.

K108, K109, and K110, were same as those in MexB and MexX overproducers, *P. aeruginosa* OCR1 and *P. aeruginosa* KG5005 (formerly N127) (13) (Table 1). On the contrary, MexC and MexE were not detected in all isolates.

Since the isolates that were resistant to LVFX had a point mutation in *gyrA* or *gyrB*, and over-expressed MexB and MexX, a complementation test was carried out to determine whether *P. aeruginosa* resistance to FQs arises from a functional cooperation between alteration of target enzymes and construction of efflux systems. Plasmids, pPAW207 and pPBW801, carrying wild-type *gyrA* and *gyrB* genes, respectively, from *E. coli* KL16 (15) were introduced into all isolates using an electroporation method (12). Introduction of plasmid pPAW207 or pPBW801 into strains that had no mutation in QRDR, i.e. K101, K102, K103, K104, K106, K111, and PAO1 resulted in no, or only a slight change in MIC of LVFX (Table 2). On the other hand, introduction of plasmid pPAW207 into strains K107, K108, K109, and K110 decreased MIC of LVFX by 8-fold. Although the decreased MICs of LVFX were still higher than those for K101 and PAO1, a reference strain, they were similar to those for K106 and K111, both of which had no mutation in *GyrA* or *GyrB* and over-expressed MexB and MexX. Since it is well known that introduction of wild-type genes into FQ-resistant *P. aeruginosa* with mutation in gyrase genes results in FQs-susceptible strains (15), our results indicate that *P. aeruginosa* resistance to FQs arises from a functional cooperation between mutation in QRDR of *gyrA* and over-expression of proteins constructing efflux systems. In general, *P. aeruginosa* strains with mutation in *gyrB* show moderate resistance to FQs and are not clinically so important (8). However in this study, when pPBW801 was introduced into K105, an 8-fold decrease in MIC of LVFX was observed, indicating that *gyrB* mutation is responsible for some resistance to FQs in K105. Considering the fact that introduction of wild-type *gyrB* gene into K105 resulted in a strain more resistant to LVFX than the fully susceptible strain, cooperation between alternation in *GyrB* and over-expression of efflux system proteins confers high resistance to FQs. Therefore, it is assumed that mutations in *gyrB* are as important as those in *gyrA* in *P. aeruginosa* resistance to FQs.

The MIC of LVFX for the K103 isolate was 2 μg/ml, which was 4-fold higher than that for K101. However, K103 had no mutations in the QRDR of DNA gyrase or topoisomerase IV genes, and did not over-express the four efflux systems tested here. It is therefore suggested that other mechanisms might be responsible for K103 resistance.

A comparison of the characteristics of K103, K105, and K108 isolated from the sputum (Table 1) indicates that alteration of target enzymes and over-expression of protein that construct efflux systems occur simultaneously during treatment and that a highly FQ-resistant strain of *P. aeruginosa* can emerge unexpectedly. Hence, in the treatment of a *P. aeruginosa* infection, a susceptibility test for isolates should be performed routinely.

We thank Dr. Hiroaki Yoshida for his critical reading of the manuscript and useful discussion. This research was partially supported by grants for scientific research to N.G. from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan and from the Ministry of Health, Labour and Welfare, Japan.

References

1) Akasaka, T., Tanaka, M., Yamaguchi, A., and Sato, K. 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. **45**: 2263–2268.
2) Anthony, M., Rose, B., Pegler, M.B., Elkins, M., Service, H., Thamotharampillai, K., Watson, J., Robinson, M., Bye, P., Merlino, J., and Harbour, C. 2002. Genetic analysis of *Pseudomonas aeruginosa* isolates from the sputa of Australian adult cystic fibrosis patients. J. Clin. Microbiol. **40**: 2772–2778.
3) Drlica, K., and Zhao, X. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61**: 377–392.
4) Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L.K., and Pechère, J.-C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23**:

345–354.
5) Kureishi, A., Diver, J.M., Beckthold, B., Schollaardt, T., and Bryan, L.E. 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **38**: 1944–1952.
6) Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44**: 3322–3327.
7) Mine, T., Morita, Y., Kataoka, A., Mizushima, T., and Tsutiya, T. 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43**: 415–417.
8) Mouneimné, H., Robert, J., Jarlier, V., and Cambau, E. 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43**: 62–66.
9) National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standards M7-A6, 6th ed, National Committee for Clinical Laboratory Standards, Wayne, Penn.
10) Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamasaki, T., Neshat, S., Yamagishi, J., Li, X.-Z., and Nishino, T. 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21**: 713–724.
11) Poole, K., Krebes, K., McNally, C., and Neshat, S. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175**: 7363–7372.
12) Smith, A.W., and Iglewski, B.H. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17**: 10509.
13) Srikumar, R., Kon, T., Gotoh, N., and Poole, K. 1998. Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. Antimicrob. Agents Chemother. **42**: 65–71.
14) Yoshida, H., Bogaki, M., Nakamura, M., and Nakamura, S. 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34**: 1271–1272.
15) Yoshida, H., Nakamura, M., Bogaki, M., and Nakamura, S. 1990. Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**: 1273–1275.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2005, p. 4760–4762 Vol. 49, No. 11
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.11.4760–4762.2005

Kinetics Study of KPC-3, a Plasmid-Encoded Class A Carbapenem-Hydrolyzing β-Lactamase

Jimena Alba,[1] Yoshikazu Ishii,[1]* Kenneth Thomson,[2] Ellen Smith Moland,[2] and Keizo Yamaguchi[1]

Department of Microbiology and Infectious Diseases, Toho University School of Medicine, 5–21–16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan,[1] and Center for Research in Anti-Infectives and Biotechnology, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, Nebraska 68178[2]

Received 8 February 2005/Returned for modification 12 March 2005/Accepted 3 August 2005

The kinetic activity of KPC-3, a plasmid-encoded class A carbapenemase, was studied. It hydrolyzed penicillins, cephalosporins, carbapenems, and even sulbactam. The best substrate was cephalothin ($k_{cat/K}m$ = 3.48 μM^{-1} s^{-1}). The efficiency of the enzyme was similar for imipenem and meropenem (k_{cat}/K_m, 1.4 and 1.94 μM^{-1} s^{-1}, respectively).

Carbapenem use has increased during the past 2 decades. This is due, in part, to their broad-spectrum of antibacterial activity and their resistance to hydrolysis by extended spectrum β-lactamases (1, 10, 14, 17). However, the appearance of carbapenemases and other carbapenem resistance mechanisms is threatening the effectiveness of this antibiotic class. In gram-negative bacteria, carbapenem resistance has been attributed to three main mechanisms: the combination of high-level production of an AmpC β-lactamase and the loss of outer membrane proteins (5, 13), changes in the affinity of penicillin binding proteins for carbapenems (7, 8), and the production of a carbapenem-hydrolyzing β-lactamase (11, 16). Although clinically significant, carbapenem-hydrolyzing β-lactamases remain rare, but their frequency has been increasing. The β-lactamases involved belong to Ambler molecular classes A, B, and D (16, 19). A small number of class A enzymes have been found to be able to hydrolyze carbapenems (6). They belong to group 2f, as defined by Bush and colleagues (4, 23). They hydrolyze ampicillin and early cephalosporins more efficiently than carbapenems and can be inhibited by clavulanic acid. Class A carbapenemases can be chromosomally encoded (NMC-A, Sme-1 to -3, IMI-1) (9, 15, 21, 22, 24, 27) or plasmid encoded (KPC-1, KPC-2, GES-2) (18, 20, 25, 26, 28, 29, 30).

KPC-type β-lactamases have become one of the most frequently encountered carbapenem-hydrolyzing enzymes on the East Coast of the United States (2). KPC-3 is the most recently reported enzyme in that group (T. Hong, E. S. Moland, B. Abdalhamid, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1–665, 2003; K. Young, P. Tierno, Jr., L. Tysall, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2–50, 2003). KPC-3 is closely related to its predecessors, differing by only 1 amino acid from KPC-2 (H272Y) and by 2 amino acids with KPC-1 (S174G, H272Y). It has been recovered from isolates of *Klebsiella pneumoniae* (Young et al., 43rd ICAAC), *Escherichia coli* (Hong et al., 43rd ICAAC), and *Enterobacter cloacae* (3).

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan. Phone: 81-3-3762-4151, ext. 2396. Fax: 81-3-5493-5415. E-mail: yoishii@med.toho-u.ac.jp.

In this study we purified KPC-3 and subjected the enzyme to kinetic characterization.

An isolate of *E. coli* (isolate 233) showing reduced susceptibility to carbapenems was referred to Creighton University from Hackensack University Medical Center. It was subsequently found to produce KPC-3 (report in press).

The isolate was grown in 4 liters of Luria-Bertani broth at 37°C (250 rpm) for 8 h, harvested, and suspended in 30 mM Tris-HCl buffer (pH 8.0) containing 30% sucrose. The periplasmic content was extracted as described previously (12). Purification was achieved using a HiPrep 16/10 SP XL column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in 10 mM acetate buffer (pH 5.0). Fractions displaying β-lactamase activity, observed as the initial rate of hydrolysis of nitrocefin (100 μM) ($\Delta\varepsilon_{482}$ = +10,000 $M^{-1}cm^{-1}$) (Oxoid Ltd., Hampshire, United Kingdom) were obtained after elution with a linear gradient of NaCl (0 to 400 mM). After concentration using Amicon ultrafiltration membranes (Millipore Corporation, Bedford, MA) and overnight dialysis in 10 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5) at 4°C, the sample was reloaded onto a Mono S HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated in MES buffer (pH 5.5), and eluted with a linear gradient of NaCl (0 to 300 mM). The entire purification process was done with an AKTA purifier (Amersham Pharmacia Biotech). The purity of the β-lactamase preparation was controlled using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The purity achieved was above 90%. The purified enzyme was then dialyzed overnight at 4°C in phosphate buffer (pH 7.0) and concentrated. Four liters of culture yielded a total of 0.27 mg of pure enzyme. After determination of the protein concentration using a Bio-Rad (Richmond, Calif.) protein assay, 20 μg/ml bovine serum albumin was added. The N-terminal sequence was determined using a Procise 492clC-1 protein sequencer (Applied Biosystems, Foster City, Calif.), and the kinetic parameters were determined with the pure enzyme.

All kinetics studies were done by measuring hydrolysis rates with a Shimadzu (Kyoto, Japan) UV-2550 spectrophotometer connected to a personal computer. To determine the kinetic parameters, 6 to 10 different concentrations of each β-lactam were used. Each reported parameter is an average of three

TABLE 1. Comparison of kinetic parameters for KPC-1, KPC-2, and KPC-3[a]

Substrate	K_m or K_i (μM)			k_{cat} (s^{-1})			k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)		
	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3
Ampicillin	130	239	65 (±5)	110	210	77 (±4)	0.9	0.9	1.2
Nitrocefin	NA[b]	NA	42 (±5)	NA	NA	107 (±5)	NA	NA	2.6
Cephaloridine	560	500	261 (±1)	340	530	364 (±4)	0.6	1.1	1.4
Cephalotin	53	82	44 (±3)	75	69	153 (±5)	1.4	0.8	3.5
Cefotaxime	160	220	95 (±8)	14	22	52 (±4)	0.1	0.1	0.5
Ceftazidime	94	NA	88 (±1)	0.1	0.1	3 (±0.06)	0.001	NA	0.03
Cefoxitin[c]	120	180	970 (±65)	0.3	0.3	0.05[d] (±0.001)	0.002	0.002	0.5[d]
Moxalactam[c]	NA	NA	14 (±1.6)	NA	NA	0.008 (±0.0002)	NA	NA	0.05
Meropenem	12	15	4 (±1)	3	4	6 (±0.09)	0.3	0.3	1.4
Imipenem	81	51	23 (±6)	12	15	45 (±0.04)	0.2	0.3	1.9
Sulbactam	NA	NA	30 (±0.9)	NA	NA	4 (±0.1)	NA	NA	0.1

[a] Kinetic values used for KPC-1 and KPC-2 were reported by H. Yigit et al. (29, 30).
[b] NA, not available.
[c] K_i values were obtained using 100 μM nitrocefin as a reporter substrate.
[d] k_{cat} values were obtained by hydrolyzing a low concentration of substrate with a high concentration of enzyme.

separate measurements. All kinetic parameters were acquired by measuring the initial hydrolysis rate of the β-lactam under study at a constant temperature of 30°C, using 50 mM phosphate buffer (pH 7.0). Analysis of the data was done using the Hanes-Woolf linearization of the Michaelis-Menten equation. For all poor substrates, the competitive inhibition constant (K_i) was determined by competition experiments between the tested β-lactam and 100 μM nitrocefin. Hydrolysis of the substrate at a concentration 10 times the K_m (K_i) value or higher yielded the catalytic constants (k_{cat}) for poor substrates. The k_{cat} value for cefoxitin was obtained using 100 μM substrate with 1.1×10^{-7} M enzyme.

The N-terminal sequence of KPC-3 was determined as LT NLVAEPFAKLE. Table 1 shows a comparison between previously reported k_{cat}, K_m (K_i), and k_{cat}/K_m values for KPC-1 and KPC-2 (29, 30) and the parameters obtained for KPC-3 in this study. KPC-3 hydrolyzed penicillins, cephalosporins, and carbapenems. Among the substrates tested, the highest hydrolytic efficiency was seen with nitrocefin and cephalothin (k_{cat}/K_m, 2.55 $\mu M^{-1}\ s^{-1}$ and 3.48 $\mu M^{-1}\ s^{-1}$, respectively). The k_{cat} and K_m values for cephaloridine were very high (k_{cat}, 364 s^{-1}; K_m, 261 μM). Imipenem and meropenem were hydrolyzed by KPC-3 with good efficiencies (k_{cat}/K_m, 1.94 $\mu M^{-1}\ s^{-1}$ and 1.40 $\mu M^{-1}\ s^{-1}$, respectively), which were similar overall to those exhibited for ampicillin and cephaloridine. The substrate profiles for the three enzymes were similar overall, although the catalytic efficiency of KPC-3 appeared to be somewhat higher with some substrates, including oxyiminocephalosporins and carbapenems. One of the notable differences is the behavior of this enzyme with ceftazidime. The catalytic activity (k_{cat}) with ceftazidime (3.0 s^{-1} ± 0.01) was approximately 30 times higher than those of KPC-1 and KPC-2 (0.1 s^{-1} for both). Due to this, KPC-3 was 30 times more efficient than KPC-1 toward this substrate. Moreover, KPC-3 showed a lower affinity for cefoxitin than the other enzymes. The present findings, therefore, suggest that the amino acid substitution that differentiates KPC-3 from KPC-2 (H272Y) could have a functional significance. A molecular modeling analysis based on the structure of the TOHO-1 enzyme (25), now called CTX-M-44, suggested that the H272Y mutation (which would be at a position similar to that of R274 in TOHO-1) could influence the positions of R209, which interacts with the substrate carboxylate. Further investigation will be necessary to clarify these matters.

We thank Tao Hong of Hackensack University Medical Center for providing us with the resistant *E. coli* strain.

J.A. was supported by a grant from the Japan Health Sciences Foundation. This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan (H15-Shinko-09) and by Toho University project research grants 15-4 and 16-6.

REFERENCES

1. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14**:933–951, table of contents.
2. **Bradford, P. A., S. Bratu, C. Urban, M. Visalli, N. Mariano, D. Landman, J. J. Rahal, S. Brooks, S. Cebular, and J. Quale.** 2004. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 β-lactamases in New York City. Clin. Infect. Dis. **39**:55–60.
3. **Bratu, S., D. Landman, M. Alam, E. Tolentino, and J. Quale.** 2005. Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter* spp. from Brooklyn, New York. Antimicrob. Agents Chemother. **49**:776–778.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39**:1211–1233.
5. **Cao, V. T., G. Arlet, B. M. Ericsson, A. Tammelin, P. Courvalin, and T. Lambert.** 2000. Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. J. Antimicrob. Chemother. **46**:895–900.
6. **Drusano, G. L., H. Lode, and J. R. Edwards.** 2000. Meropenem: clinical response in relation to in vitro susceptibility. Clin. Microbiol. Infect. **6**:185–194.
7. **Edwards, R., and D. Greenwood.** 1996. Mechanisms responsible for reduced susceptibility to imipenem in *Bacteroides fragilis*. J. Antimicrob. Chemother. **38**:941–951.
8. **Fernandez-Cuenca, F., L. Martinez-Martinez, M. C. Conejo, J. A. Ayala, E. J. Perea, and A. Pascual.** 2003. Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. J. Antimicrob. Chemother. **51**:565–574.
9. **Henriques, I., A. Moura, A. Alves, M. J. Saavedra, and A. Correia.** 2004. Molecular characterization of a carbapenem-hydrolyzing class A β-lactamase, SFC-1, from *Serratia fonticola* UTAD54. Antimicrob. Agents Chemother. **48**:2321–2324.
10. **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.
11. **Livermore, D. M., and N. Woodford.** 2000. Carbapenemases: a problem in waiting? Curr. Opin. Microbiol. **3**:489–495.
12. **Ma, L., J. Alba, F.-Y. Chang, M. Ishiguro, K. Yamaguchi, L. K. Siu, and Y. Ishii.** 2005. Novel SHV-derived extended-spectrum β-lactamase, SHV-57, that confers resistance to ceftazidime but not cefazolin. Antimicrob. Agents Chemother. **49**:600–605.
13. **Martinez-Martinez, L., A. Pascual, S. Hernandez-Alles, D. Alvarez-Diaz,**

A. I. Suarez, J. Tran, V. J. Benedi, and G. A. Jacoby. 1999. Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **43**:1669–1673.

14. **Mushtaq, S., Y. Ge, and D. M. Livermore.** 2004. Comparative activities of doripenem versus isolates, mutants, and transconjugants of *Enterobacteriaceae* and *Acinetobacter* spp. with characterized β-lactamases. Antimicrob. Agents Chemother. **48**:1313–1319.
15. **Nordmann, P., S. Mariotte, T. Naas, R. Labia, and M. H. Nicolas.** 1993. Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*. Antimicrob. Agents Chemother. **37**:939–946.
16. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8**:321–331.
17. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46**:1–11.
18. **Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In*52* from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44**:622–632.
19. **Poirel, L., and P. Nordmann.** 2002. Acquired carbapenem-hydrolyzing β-lactamases and their genetic support. Curr. Pharm. Biotechnol. **3**:117–127.
20. **Poirel, L., G. F. Weldhagen, T. Naas, C. De Champs, M. G. Dove, and P. Nordmann.** 2001. GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45**:2598–2603.
21. **Pottumarthy, S., E. S. Moland, S. Juretschko, S. R. Swanzy, K. S. Thomson, and T. R. Fritsche.** 2003. NmcA carbapenem-hydrolyzing enzyme in *Enterobacter cloacae* in North America. Emerg. Infect. Dis. **9**:999–1002.
22. **Queenan, A. M., C. Torres-Viera, H. S. Gold, Y. Carmeli, G. M. Eliopoulos, R. C. Moellering, Jr., J. P. Quinn, J. Hindler, A. A. Medeiros, and K. Bush.** 2000. SME-type carbapenem-hydrolyzing class A β-lactamases from geographically diverse *Serratia marcescens* strains. Antimicrob. Agents Chemother. **44**:3035–3039.
23. **Rasmussen, B. A., and K. Bush.** 1997. Carbapenem-hydrolyzing β-lactamases. Antimicrob. Agents Chemother. **41**:223–232.
24. **Rasmussen, B. A., K. Bush, D. Keeney, Y. Yang, R. Hare, C. O'Gara, and A. A. Medeiros.** 1996. Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **40**:2080–2086.
25. **Shimamura, T., A. Ibuka, S. Fushinobu, T. Wakagi, M. Ishiguro, Y. Ishii, and H. Matsuzawa.** 2002. Acyl-intermediate structures of the extended-spectrum class A β-lactamase, Toho-1, in complex with cefotaxime, cephalothin, and benzylpenicillin. J. Biol. Chem. **277**:46601–46608.
26. **Smith Moland, E., N. D. Hanson, V. L. Herrera, J. A. Black, T. J. Lockhart, A. Hossain, J. A. Johnson, R. V. Goering, and K. S. Thomson.** 2003. Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J. Antimicrob. Chemother. **51**:711–714.
27. **Vourli, S., P. Giakkoupi, V. Miriagou, E. Tzelepi, A. C. Vatopoulos, and L. S. Tzouvelekis.** 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. **234**:209–213.
28. **Yang, Y. J., P. J. Wu, and D. M. Livermore.** 1990. Biochemical characterization of a β-lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. Antimicrob. Agents Chemother. **34**:755–758.
29. **Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-Sanchez, J. W. Biddle, C. D. Steward, S. Alberti, K. Bush, and F. C. Tenover.** 2001. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45**:1151–1161.
30. **Yigit, H., A. M. Queenan, J. K. Rasheed, J. W. Biddle, A. Domenech-Sanchez, S. Alberti, K. Bush, and F. C. Tenover.** 2003. Carbapenem-resistant strain of *Klebsiella oxytoca* harboring carbapenem-hydrolyzing β-lactamase KPC-2. Antimicrob. Agents Chemother. **47**:3881–3889.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 458–461
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.458–461.2005

Vol. 43, No. 1

Clonal Diversity of Metallo-β-Lactamase-Possessing *Pseudomonas aeruginosa* in Geographically Diverse Regions of Japan

Soichiro Kimura,[1] Jimena Alba,[1] Katsuaki Shiroto,[1] Reiko Sano,[2] Yoshihito Niki,[3] Shigefumi Maesaki,[4] Koji Akizawa,[5] Mitsuo Kaku,[6] Yuji Watanuki,[7] Yoshikazu Ishii,[1]* and Keizo Yamaguchi[1]

Department of Microbiology, Toho University School of Medicine, Tokyo[1]; Nara Medical University, Nara[2]; Kawasaki Medical School, Okayama[3]; Saitama Medical School, Saitama[4]; Hokkaido University Hospital, Sapporo[5]; Tohoku University Graduate School of Medicine, Miyagi[6]; and Kanagawa Cardiovascular and Respiratory Diseases Center, Kanagawa,[7] Japan

Received 24 March 2004/Returned for modification 30 April 2004/Accepted 1 June 2004

The aim of this study was to determine the distribution of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in Japan and to investigate the molecular characteristics of resistance gene cassettes including the gene encoding this enzyme. A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in 2002 were evaluated. This study indicated that although the prevalence of imipenem-resistant *P. aeruginosa* has not increased compared to that found in previous studies, clonal distribution of the same strain across Japan is evident.

Class A, B, and D β-lactamases, as defined by Ambler et al., can hydrolyze carbapenems (1, 9). In particular, class B β-lactamases, termed metallo-β-lactamases, are an increasingly serious clinical problem because they have a very broad substrate profile that includes penicillins, expanded-spectrum cephalosporins, and carbapenems and excludes only monobactams, such as aztreonam. It has been reported that IMP-1 metallo-β-lactamase-producing *Serratia marcescens* was first isolated in Japan in 1991 (10). Recently, metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *S. marcescens* probably have the highest incidence of isolation in Japan (7).

Most metallo-β-lactamase genes are located on integrons, which are genetic elements containing gene cassettes that can facilitate their spread and mobilize the genes to other integrons or to other sites. The gene cassettes often encode clinically important antibiotic resistance genes, including those encoding β-lactamases such as extended-spectrum β-lactamases and carbapenemases, and also aminoglycoside-modifying enzymes (12).

Little is known about the distribution of the clone(s) that produces metallo-β-lactamases in Japan. Therefore, we conducted a surveillance study covering a wide geographic area with the aim of determining the distribution of metallo-β-lactamase producers in Japan and to investigate the molecular characteristics of the resistance gene cassettes that included the gene encoding a metallo-β-lactamase.

A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in the year 2002 were evaluated. The susceptibility of *P. aeruginosa* to several antibiotics was measured with the Etest strip, and the strains were stored on Casitone medium (Eiken Chemical Co. Ltd., Tokyo, Japan) (data not shown). After 6 months, the antibiotic susceptibility of these isolates was reassessed by the National Committee for Clinical Laboratory Standards broth microdilution method with cation-adjusted Mueller-Hinton broth (Difco, Detroit, Mich.). The isolates were screened for the presence of metallo-β-lactamase by a double-disk synergy test reported by Arakawa et al. (2). Integron analysis was performed by PCR mapping (5′-conserved segment *int*I to 3′-conserved segment *qacEΔ1*) of the typical antibiotic resistance genes and integron with specific primer sets (Table 1). The specificity of the primer sets for bla_{IMP-1}-like and bla_{VIM-2}-like gene was confirmed with positive-control strains producing IMP-1 or VIM-2 metallo-β-lactamase. The specificity of amplicons obtained by specific primer sets (*aacA4*, *aadA1*, *aadA2*, and bla_{OXA-2}) was also partially verified with the automatic sequencer ABI Prism 310 genetic analyzer (Applied Biosystems/Perkin-Elmer Biosystems). PCR with Ex *Taq* polymerase (Takara Bio, Inc., Tokyo, Japan) were carried out by standard methodology (13). pulsed-field gel electrophoresis analysis was performed by a modified method of the standard protocol (6). The restriction enzyme used was SpeI (15). By use of the dendrogram, isolates with a genetic relatedness of >80% were

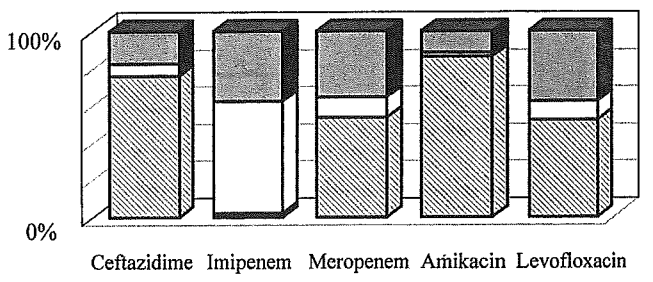

FIG. 1. Antimicrobial susceptibilities of imipenem-nonsusceptible *P. aeruginosa* isolates.

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan. Phone: 81-3-3762-4151, ext. 2396. Fax: 81-3-5493-5415. address: E-mail: yoishii@med.toho-u.ac.jp.

TABLE 1. Nucleotide sequences of PCR primers used in this study

Gene[a]	Primer sequence (5′ to 3′)	T_m (°C)	Reference
*int*A(S)	ATC ATC GTC GTA GAG ACG TCG G	67.4	11
*int*B(AS)	GTC AAG GTT CTG GAC CAG TTG C	66.9	11
bla_{IMP-1}(S)	CTA CCG CAG CAG AGT CTT TG	62.7	This study
bla_{IMP-1}(AS)	AAC CAG TTT TGC CTT ACC AT	59.9	This study
bla_{VIM-2}(S)	AAA GTT ATG CCG CAC TCA CC	63.9	This study
bla_{VIM-2}(AS)	TGC AAC TTC ATG TTA TGC CG	64.5	This study
aacA4(S)	GAC CTT GCG ATG CTC TAT GAG TGG CTA AAT	73.0	This study
aacA4(AS)	TTC GCT CGA ATG CCT GGC GTG TT	76.9	This study
aadA1(S)	TGA TCG CCG AAG TAT CGA CTC	66.3	This study
aadA1(AS)	CCT TGG TGA TCT CGC CTT TC	65.8	This study
aadA2(S)	TTC GAA CCA ACT ATC AGA GGT GCT AA	67.4	This study
aadA2(AS)	AAA GCG AAT AAA TTC TTC CAA GTG ATC T	66.4	This study
bla_{OXA-2}(S)	CAA TCC GAA TCT TCG CGA TAC TT	66.9	This study
bla_{OXA-2}(AS)	AAG TAT CGC GAA GAT TCG GAT TG	66.9	This study
qacEΔ1	CTC TCT AGA TTT TAA TGC GGA TG	60.6	This study

[a] (S), sense; (AS), antisense.

considered to represent the same pulsed-field gel electrophoresis type (4).

Eighty-eight (15%) of 594 isolates were not susceptible (MIC ≥ 8 mg/ml) to imipenem. Among 88 isolates, 88 (100%), 21 (24%), 41 (47%), 12 (14%), and 42 (48%) were not susceptible to imipenem, ceftazidime, meropenem, amikacin, and levofloxacin, respectively (Fig. 1). Screening of metallo-β-lactamase producers was carried out for these isolates by the double-disk synergy test. Eleven (1.9%) of 594 isolates were found to produce metallo-β-lactamase. Ten of these isolates were IMP-1-like, and the other was a VIM-2-like metallo-β-lactamase producer.

The type of metallo-β-lactamase gene was also confirmed by PCR. The genetic relatedness of these isolates was also evaluated by pulsed-field gel electrophoresis as described above (Fig. 2, Table 2). Strains TUM1683, TUM1708, TUM1709, TUM1710, and TUM1732 had related electrophoresis chromosomal DNA banding patterns, whereas other strains (TUM1672, TUM1673, TUM1682, TUM1721, TUM1733, and TUM1757) showed different banding patterns. Strain TUM1708, TUM1709, and TUM1710 were isolated from same hospital, suggesting nosocomial spread. Interestingly, although strains TUM1683, TUM1708 (or TUM1709 and TUM1710), and TUM1732 has been isolated in different hospitals, Kawasaki, Saitama, and Nara, respectively, these isolates had related patterns. Since the distance from Okayama to Saitama and from Saitama to Nara is about 800 and 400 km, respectively, the results observed suggested clonal spread of metallo-β-lactamase-producing strains.

Several researchers have reported an incidence of metallo-β-lactamase-producing *P. aeruginosa* of between 0.4 and 1.3% in Japan from 1992 to 2002 (5, 7, 14, 16). In this study, we isolated 1.9% metallo-β-lactamase-producing *P. aeruginosa* strains from geographically diverse regions in Japan. We suggest that the incidence of metallo-β-lactamase-possessing *P. aeruginosa* has not increased during the past decade. However, the same clone of metallo-β-lactamase-carrying *P. aeruginosa* has now spread throughout Japan.

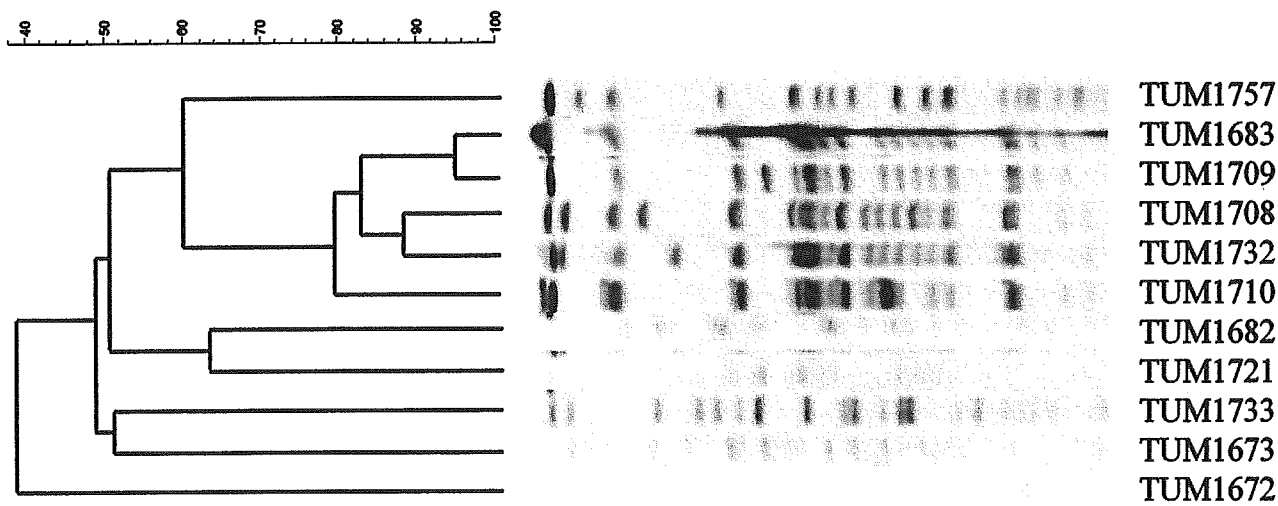

FIG. 2. Pulsed-field gel electrophoresis profiles obtained with SpeI chromosomal digestion of metallo-β-lactamase-carrying *P. aeruginosa*. The second through sixth lanes contained related strains TUM1683, TUM1709, TUM1708, TUM1732, and TUM1710, respectively. Lanes first and seventh to eleventh lanes contained unrelated strains TUM1757, TUM1682, TUM1721, TUM1733, TUM1673, and TUM1672, respectively.

TABLE 2. Characteristics of bla_{IMP}-containing non-imipenem-susceptible *P. aeruginosa* isolates

Strain	Hospital no.	Material	Type of enzyme	Pattern[b]	Integron structure[c]	MIC (μg/ml)[a]									
						CAZ	IPM	MEM	LVX	AZT	AMK	NET	GEN	KAN	ABK
TUM1672	1	Urine	VIM-2-like	A	I	64	>128	>128	16	32	0.06	0.5	0.5	8	0.06
TUM1673	1	Sputum	IMP-1-like	B	II	>128	8	32	16	8	64	>128	4	>128	16
TUM1682	2	Sputum	IMP-1-like	C	III	>128	64	>128	32	32	32	>128	2	>128	2
TUM1683	2	Sputum	IMP-1-like	D	IV	>128	64	>128	32	64	16	>128	2	>128	2
TUM1708	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	4
TUM1709	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	2
TUM1710	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	64	32	>128	2	>128	4
TUM1721	4	Urine	IMP-1-like	E	V	>128	64	>128	32	32	32	>128	>128	>128	64
TUM1732	5	Urine	IMP-1-like	D	IV	>128	64	>128	32	128	32	>128	4	>128	2
TUM1733	5	Pus	IMP-1-like	F	VI	>128	64	>128	64	32	2	>128	>128	>128	1
TUM1757	6	Sputum	IMP-1-like	G	VII	>128	64	>128	16	16	32	>128	1	>128	16

[a] CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; AZT, aztreonam; AMK, amikacin; NET, netilmicin; GEN, gentamicin; KAN, kanamycin; ABK, arbekacin.

[b] PEGE profiles obtained with SpeI chromosomal digestion of *P. aeruginosa* carrying a metallo-β-lactamase gene as recommended by Tenover et al. (15).

[c] Integron structures possessed by each gene as mentioned in the text. I, bla_{VIM-2}-like, *aacA4* and *aadA2*; II, bla_{IMP-1}-like, *aadA1* and *orfG*; III, bla_{IMP-1}-like, *aadA1* and unknown gene; IV, bla_{IMP-1}-like, *aadA1* and unknown gene; V, bla_{IMP-1}-like, *aacA4*, *aadA1* and bla_{OXA-2}; VI, bla_{IMP-1}-like, *aacA4*; VII, only bla_{IMP-1}-like gene.

It has been reported that genetic analysis of bla_{IMP-1} revealed features typical of an integron-located gene (9). The detection of a type 1 integron was confirmed in 11 strains. In these strains, bla_{IMP-1}-like or bla_{VIM-2}-like genes were located immediately downstream of the *IntI*1 integrase gene. However, these isolates possessed a variety of gene cassettes, such as the *aacA4* aminoglycoside 6′-*N*-acetyltransferase gene and *aadA1* and *aadA2* aminoglycoside adenyltransferase genes between the metallo-β-lactamase gene and *qacΔE1*. Therefore, these isolates are likely resistant not only to β-lactams but also to aminoglycosides. Interestingly, strain TUM1721 possessed not only the bla_{IMP-1}-like genes *aacA4* and *aadA1* but also an OXA-type β-lactamase gene on the integron gene cassette.

Little is known about optimal chemotherapy for infection due to metallo-β-lactamase-producing *P. aeruginosa*. To detail the antibiotic susceptibility of *P. aeruginosa* possessing a metallo-β-lactamase, the MICs of several antibiotics were evaluated (Table 2). All of the isolates were resistant to ceftazidime, meropenem, and levofloxacin. Ten of the 11 were resistant to imipenem and netilmicin, nine were resistant to aztreonam, and eight were not susceptible to amikacin. Bellais et al. reported that chemotherapy with high aztreonam doses effectively reduced viable cells of a metallo-β-lactamase-producing strain of *P. aeruginosa* in a rat pneumonia model (3). In general, although metallo-β-lactamases do not hydrolyze aztreonam, 9 of 11 isolates were resistant to aztreonam in this study (MIC ≥ 32 μg/ml). On the other hand, arbekacin was found to suppress the growth of some isolates in this study. In Japan, arbekacin, which has fewer side effects than vancomycin, has been used against methicillin-resistant *Staphylococcus aureus* (8). Recently, arbekacin-resistant *P. aeruginosa* possessing the 16S rRNA methylase gene *rmtA* was isolated in Japan (17). However, the incidence of these isolates is still low (0.8%, 9 of 1,113 clinical isolates). Therefore, arbekacin could be used as treatment against metallo-β-lactamase-possessing *P. aeruginosa*.

In conclusion, this study indicates that although the prevalence of metallo-β-lactamase-producing *P. aeruginosa* has not increased, this pathogen has spread from a single source to a wide geographic area of Japan. Further surveillance and monitoring of multidrug-resistant *P. aeruginosa* should be a high priority.

This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan during 2003 (H15-Iyaku-003 and H15-shinkou-009). S.K. was supported by a grant from the Society of Japanese Pharmacopoeia.

We thank Kenneth S. Thomson, Creighton University School of Medicine, for useful advice. We also thank Kunimoto Hotta, National Institute of Infectious Diseases, for helpful discussions.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Phil. Trans R. Soc. Lond. B Biol. Sci. **289**:321–331.
2. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38**:40–43.
3. **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. **Duck, W. M., C. D. Steward, S. N. Banerjee, J. E. McGowan, Jr., and F. C. Tenover.** 2003. Optimization of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. J. Clin. Microbiol. **41**:3035–3042.
5. **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.
6. **Ishii, Y., J. Alba, S. Kimura, K. Nakashima, Y. Abe, and K. Yamaguchi.** 2002. Rapid pulsed-field gel electrophoresis technique for determination of genetic diversity of *Serratia marcescens*. J. Infect. Chemother. **8**:368–370.
7. **Kurokawa, H., T. Yagi, N. Shibata, K. Shibayama, and Y. Arakawa.** 1999. Worldwide proliferation of carbapenem-resistant gram-negative bacteria. Lancet **354**:955.
8. **Matsuo, H., M. Kobayashi, T. Kumagai, M. Kuwabara, and M. Sugiyama.** 2003. Molecular mechanism for the enhancement of arbekacin resistance in a methicillin-resistant *Staphylococcus aureus*. FEBS Lett. **546**:401–406.
9. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8**:321–331.
10. **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.
11. **Rosser, S. J., and H. K. Young.** 1999. Identification and characterization of class 1 integrons in bacteria from an aquatic environment. J. Antimicrob. Chemother. **44**:11–18.
12. **Rowe-Magnus, D. A., and D. Mazel.** 2002. The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. **292**:115–125.
13. **Sambrook, J., and D. W. Russell.** 2001. In vitro amplification of DNA by the polymerase chain reaction, p. 8.1–8.126. In J. Sambrook and D. W. Russell (ed.), Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

14. **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.
15. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
16. **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.
17. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa.* Lancet **362:**1888–1893.

JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2005, p. 1072–1075
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.3.1072–1075.2005

Vol. 43, No. 3

Extended-Spectrum β-Lactamase-Producing Shiga Toxin Gene (stx_1)-Positive *Escherichia coli* O26:H11: a New Concern

Yoshikazu Ishii,[1]* Soichiro Kimura,[1] Jimena Alba,[1] Katsuaki Shiroto,[1] Masanobu Otsuka,[2] Naotaka Hashizume,[2] Kazumichi Tamura,[3] and Keizo Yamaguchi[1]

Department of Microbiology, Toho University School of Medicine,[1] Department of Clinical Laboratory, Toho University Ohashi Hospital,[2] and Department of Bacteriology, National Institute of Infectious Diseases,[3] Tokyo, Japan

Received 4 August 2004/Returned for modification 24 September 2004/Accepted 3 October 2004

***Escherichia coli* strain TUM2139 was isolated from a stool sample from a 9-year-old girl on 16 June 2004. This strain was categorized as Shiga toxin-producing *Escherichia coli* (STEC) because the Shiga-like toxin gene stx_1 was detected by immunochromatography and PCR assay. The strain was highly resistant to cefotaxime (256 μg/ml) and was also resistant to cefepime, cefpodoxime, ceftriaxone, and aztreonam. In the presence of 4 μg of clavulanic acid per ml, the MIC of cefotaxime decreased to ≤0.12 μg/ml, indicating that this strain was an extended-spectrum β-lactamase (ESBL) producer. Cefotaxime resistance was transferred to *E. coli* C600 by conjugation at a frequency of 3.0×10^{-6}. A PCR assay was performed with primer sets specific for TEM-type and SHV-type ESBLs and for the CTX-M-2 (Toho-1), CTX-M-3, and CTX-M-9 groups of ESBLs. A specific signal was observed with the primer set specific for the CTX-M-9 group of β-lactamases. This β-lactamase was confirmed to be the ESBL CTX-M-18 by DNA sequencing. This is the first report of an ESBL-producing STEC isolate.**

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an important cause of waterborne and food-borne illnesses. STEC is ingested most commonly with undercooked ground beef (22). Human infection with STEC is potentially fatal and may be associated with serious complications such as hemolytic-uremic syndrome (HUS) and hemorrhagic colitis (21). The production of Stx is the unifying feature of all STEC strains. Various types of Stxs are produced, but they fall into two main types: Stx1 and Stx2 (4, 5, 6, 8). The clinical significance of four serological or biological variants of Stx2 (Stx2c, Stx2d, Stx2e, and Stx2f) is unknown (17). The majority of Stx genes are encoded by bacteriophages. The most severe or important pathogen among STEC strains is *E. coli* O157:H7. The cases of HUS in approximately 90% of children in the developed part of the world are associated with infections caused by Stx-producing bacteria; among these, 70% are caused by *E. coli* O157:H7 (21). Epidemiologic data suggest that isolates that produce Stx2 alone are more likely to cause severe disease than those that produce only Stx1 or a combination of Stx1 and Stx2 (20).

Recently, Schroeder et al. (23) reported on the antimicrobial resistance of 752 STEC strains from animals and humans. They found that 50% of *E. coli* O26, O103, O111, O128, and O145 strains from humans were resistant to ampicillin, cephalothin, tetracycline, streptomycin, or sulfamethoxazole but that no strain was resistant to expanded-spectrum β-lactams, including cefotaxime, cefpodoxime, or aztreonam. A recent study suggested that antibiotic therapy for the early stage of STEC infection is able to prevent progression of the disease to HUS (24). However, antimicrobial therapy for STEC infection is still regarded as controversial, because antibiotics induce increased levels of Stx production in vivo (18). On the other hand, many patients with diarrhea receive empirical antibiotic therapy (21).

E. coli TUM2139 was isolated from a clinical stool specimen at Toho University Ohashi Hospital. This strain was resistant to cefotaxime. On the other hand, the O-antigen type of this strain was determined to be O26. In this study, the resistance of *E. coli* TUM2139 to β-lactam antibiotics and the presence of virulence factors in this strain were confirmed by PCR assay and the direct sequencing technique.

MATERIALS AND METHODS

Bacterial strain, media, and culture. On 16 June 2004, *E. coli* TUM2139 was isolated from a stool sample from a 9-year-old female patient at Toho University Ohashi Hospital. She had diarrhea, vomiting, and a low-grade fever. The strain was identified with the Phoenix system (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan). *E. coli* C600 (F⁻ *lacY1 leuB6 supE44 thi-1 thr-1 tonA21* nalidixic acid resistant) was used as the recipient strain for the conjugation experiments. The bacterial strains were stored at −70°C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol. Subsequently, the bacterial strains were inoculated onto Mueller-Hinton agar plates and incubated overnight at 35°C.

Serotyping. *E. coli* TUM2139 was screened for Vero toxin (Stx) with a Capillia O157 immunochromatography assay kit for Vero toxin (Nippon Becton Dickinson Company, Ltd.). The O- and H-antigen types of the strain were determined with neutralizing antisera. A total of 181 types of antisera against the O antigen (O1 to O181) and also 56 different kinds of antisera against the H antigen were used. These antisera were made by the National Institute of Infectious Diseases, Tokyo, Japan, which is the typing center for *E. coli* in Japan.

PCR assay for stx_1, stx_2, *eaeA*, and *hlyA*. The use of multiplex PCR for the detection of stx_1, stx_2, *eaeA*, and enterohemorrhagic *E. coli hlyA* was reported by Fagan et al. (18). DNA amplification was performed by a PCR method directly with the colonies (26). A small amount of the test organism was picked up with a toothpick and transferred directly to 50 μl of the PCR mixture. The primers and the predicted sizes of the PCR amplicons are listed in Table 1. PCR assays were performed with EX*taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) and a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, Conn.). The PCR

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, 1438540 Tokyo, Japan. Phone: 81-3-3762-4151. Fax: 81-3-5493-5415. E-mail: yoishii@med.toho-u.ac.jp.

TABLE 1. Primer sequences and predicted lengths of PCR amplification products

Primer specificity	Direction	Primer sequence (5′ to 3′)	Fragment size (bp)	Reference or source
Virulence factors[a]				
stx_1	Sense	ACGATGTGGTTTATTCTGGA	165	8
	Antisense	CTTCACGTGACCATACATAT		
stx_2	Sense	ACACTGGATGATCTCAGTGG	614	10
	Antisense	CTGAATCCCCCTCCATTATG		
EHEC *hlyA*	Sense	CCATGACAACGGACAGCAGTT	779	10
	Antisense	CCTGTCAACTGAGCAGCACTTTG		
eaeA	Sense	GTGGCGAATACTGGCGAGACT	890	9
	Antisense	CCCCATCTTTTTCACCGTCG		
β-Lactamases[a]				
CTX-M-2	Sense	GCGAACAGCGTGCAACAGCAGCTGG	521	This study
	Antisense	GCCAGCGCTTTACCCAGCGTCAG		
CTX-M-3	Sense	GAGCATATGGTTAAAAAATCACTGCGTCAGTTC	891	This study
	Antisense	CAGGGATCCTTACAAACCGTCGGTGACGATTTTAGCC		
CTX-M-9	Sense	GTTTGAGCATATGGTGACAAAGAGAGGCAACGG	895	11
	Antisense	CAGGGATCCTTACAGCCCTTCGGCGATG		
TEM	Sense	GGGGAGCTCATAAAATTCTTGAAGAC	1,199	23
	Antisense	GGGGGATCCTTACCAATGCTTAATCA		
SHV	Sense	GTTCATATGCGTTATATTCGCCTGTG	876	This study
	Antisense	ATAGGATCCTTAGCGTTGCCAGTGCT		
CTX-M-18[b]				11
	Sense	AGAGAGTGCAACGGATGATGTT		
	Sense	GTTGCAGTACAGCGACAATACC		
	Sense	GCTGGTTCTGGTGACCTATTTTAC		
	Antisense	GCCATAACTTTACTGGTACTGCAC		
	Antisense	CTGGGTAAAATAGGTCACCAGAAC		

[a] Primers for PCR.
[b] Primers for sequencing. DNA template amplified by primers for CTX-M-9 was used for sequencing.

conditions were as follows: incubation 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s, with a final cycle of 72°C for 7 min. The amplified DNA fragments of the target genes were identified by electrophoresis on a 2% agarose gel. After electrophoresis, the gels were stained with 0.5 mg of ethidium bromide per ml and visualized with UV illumination.

Antimicrobial susceptibility testing. MICs were determined by a broth microdilution method with cation-adjusted Mueller-Hinton broth. The dilution ranges of the antibiotic agents were 0.25 to 512 μg/ml. Quality control was done by using *E. coli* ATCC 25922 as a reference strain for antibiotic susceptibility. All procedures were done and the results were interpreted as described by the National Committee for Clinical Laboratory Standards (15, 16). The organisms were inoculated at about 5×10^5 cells per well by using a MIC2000 inoculation device (Dynatech, McLean, Va.). The MIC was defined as the lowest concentration that prevented visible growth after incubation for 18 h at 35°C.

Antibiotics. The following agents, all with known potencies, were used in this study: piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan); cephalothin (Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan); cefoxitin, imipenem, and ciprofloxacin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan); ceftazidime and clavulanic acid (Glaxo Smith Kline Ltd., Tokyo, Japan); cefotaxime (Aventis Japan Ltd., Tokyo, Japan); aztreonam (Eisai Co., Ltd., Tokyo, Japan); tazobactam (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan); cefepime (Bristol Pharmaceutical Co., Ltd., Tokyo, Japan); cefotetan (Yamanouchi Pharmaceuticals Co. Ltd., Japan); cefmetazole and cefpodoxime (Sankyo Co., Ltd., Tokyo, Japan); ceftriaxone (Roche Japan K.K., Tokyo, Japan); faropenem (Suntory Ltd., Tokyo, Japan); gentamicin (Nihon Shering K.K. Osaka, Japan); kanamycin (Meiji Seika Ltd., Tokyo, Japan); minocycline (Wyeth K.K. Tokyo, Japan); and nalidixic acid (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan).

Conjugation experiment. Conjugation experiments were performed by the broth method (11). A nalidixic acid-resistant marker was added to recipient cells (*E. coli* C600). Then, the recipient cells and the donor cells (*E. coli* TUM2139) were mixed in a ratio of 1:9. The same volume of fresh LB broth was added to the cell mixture and the mixture was incubated for 90 min at 35°C. After 90 min, the cells were plated onto LB agar plates containing 5 μg of cefotaxime per ml and 25 μg of nalidixic acid per ml and incubated at 35°C overnight. Donor cells were also plated on LB agar in the absence of antibiotics. After incubation, the colonies were counted and the frequency of conjugation of a plasmid was calculated.

PCR assay for ESBLs and ESBL DNA sequence analysis. The total DNA template was obtained and the amplification products were identified by the same procedure used for the PCR assay of the bla_{TEM} type, bla_{SHV} type, bla_{Toho-1} group, $bla_{CTX-M-3}$ group, and $bla_{CTX-M-9}$ group of extended-spectrum β-lactamases (ESBLs). The primers used for PCR, the predicted sizes of the PCR amplicons, and the primers used for sequencing are listed in Table 1. PCR conditions were as follows: incubation at 94°C for 3 min and 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min), with a final extension at 72°C for 7 min.

Both strands of the PCR products obtained were sequenced by using the same set of primers used to obtain the PCR amplification products and then by using primers synthesized by primer walking. All primers used for PCR and DNA sequencing are listed in Table 1. DNA sequencing was carried out with a BigDye (version 3.1) Terminator Cycle Sequencing kit and a model 310 DNA sequencer (Applied Bio-

TABLE 2. Antimicrobial susceptibilities of donor, recipient, and conjugant strains

Antibiotic	MIC (μg/ml)		
	E. coli TUM2319	Recipient	Conjugant
Piperacillin	128	≤0.25	64
Piperacillin-tazobactam	2/4	≤0.25/4	1/4
Cephalothin	512	0.5	512
Cefoxitin	4	≤0.25	2
Cefmetazole	1	≤0.25	1
Cefotaxime	256	≤0.25	32
Cefotaxime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Ceftazidime	2	≤0.25	2
Ceftazidime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Cefpodoxime	256	≤0.25	128
Ceftriaxone	256	≤0.25	128
Cefotetan	≤0.25	≤0.25	≤0.25
Cefepime	8	≤0.25	4
Aztreonam	8	≤0.25	8
Faropenem	≤0.25	≤0.25	≤0.25
Imipenem	≤0.25	≤0.25	≤0.25
Gentamicin	≤0.25	≤0.25	≤0.25
Kanamycin	2	≤0.25	≤0.25
Minocyclin	≤0.25	≤0.25	≤0.25
Nalidixic acid	4	64	64
Ciprofloxacin	≤0.25	≤0.25	≤0.25

systems, Foster City, Calif.). The deduced amino acid sequences were examined by using the BLAST program at the DNA Data Bank of Japan (Shizuoka, Japan).

RESULTS

Characterization of *E. coli* TUM2319. *E. coli* TUM2319 was confirmed to be a O26:H11 strain with antisera. The Capillia O157 immunochromatography assay kit was used to determine whether the Vero toxin (Stx) produced by this strain is Vero toxin 1 (VT1; Stx1) and/or VT2 (Stx2). After this screening, the strain was investigated for the presence of the stx_1 and stx_2 genes by the PCR assay directly with the bacterial colonies. Only the stx_1 gene was amplified. The stx_2 gene was not detected in this strain. The *eaeA* and enterohemorrhagic *E. coli* (EHEC) *hlyA* genes were also detected by PCR. From these results, *E. coli* TUM2319 was confirmed to be an STEC strain.

Antimicrobial susceptibility testing of *E. coli* TUM2319. The antimicrobial susceptibility testing results for *E. coli* TUM2319 are presented in Table 2. *E. coli* TUM2319 was resistant to piperacillin, cephalothin, cefotaxime, ceftriaxone, and cefpodoxime but was susceptible to all other agents tested. The MIC of cefotaxime was reduced from 256 to ≤ 0.25 μg/ml in the presence of 4 μg of clavulanic acid per ml.

Conjugation experiments. Conjugation experiments showed that conjugants grew on LB agar plates in the presence of 25 μg of nalidixic acid per ml and 5 μg of cefotaxime per ml. The frequency of conjugation was 3.0×10^{-6}. The MICs of piperacillin, cephalothin, cefotaxime, cefpodoxime, and ceftriaxone for the conjugants were significantly increased compared with those for *E. coli* C600, the recipient strain (Table 2). These results show that this resistance marker could move from a resistant strain to a sensitive strain by conjugation. *E. coli* TUM2319 and its conjugants harbored a plasmid of the same size, approximately 80 kbp (data not shown).

Type of ESBL. A class A β-lactamase gene of the CTX-M-9 group was detected by PCR in *E. coli* TUM2319; but genes for ESBLs of the TEM type, SHV type, Toho-1 (CTX-M-2) group, and CTX-M-3 group were not detected. DNA sequencing confirmed that the enzyme of the CTX-M-9 group was the CTX-M-18 ESBL. This β-lactamase gene was also detected on an 80-kbp plasmid in the conjugant. Moreover, the EHEC *hlyA* gene was detected in the conjugants.

DISCUSSION

E. coli strain TUM2319 was confirmed to be an O26:H11 strain and an stx_1-, *eaeA*-, and EHEC *hlyA*-positive but stx_2-negative STEC strain by serological tests and PCR assays. *E. coli* O157:H7 infection in humans sometimes leads to HUS; however, almost all *E. coli* O26:H11 infections are mild (5).

Antibiotic treatment for STEC infections is not recommended (18). However, chemotherapy might be initiated before the diagnosis of an STEC infection. It is most widely accepted, however, that empirical therapy with antibiotics be started for children with acute diarrhea. β-Lactam antibiotics, especially expanded-spectrum β-lactams, such as cefotaxime, ceftazidime, ceftriaxone, and cefoperazone, are the most useful clinically because they combine safety with high potency against gram-negative bacteria, such as members of the family *Enterobacteriaceae*, including *E. coli*. Accordingly, expanded-spectrum β-lactams are one of the groups of antibiotics recommended for the treatment of serious *E. coli* infections (21).

Expanded-spectrum β-lactams are very stable to class A, class C, and class D β-lactamases of the Ambler classification (2); however, ESBLs can easily hydrolyze this group of β-lactam antibiotics, such as cefpodoxime, ceftriaxone, cefotaxime, and ceftazidime (7). In Japan, CTX-M-type β-lactamase-producing *E. coli* strains, such as cefotaxime-resistant *E. coli*, are often isolated from clinical specimens, with the CTX-M-2 or CTX-M-18 β-lactamases becoming the most commonly encountered ESBLs (11, 14, 25, 28, 29). The CTX-M-18 enzyme, which was initially reported to be Toho-3 (GenBank accession number AB038771) when the sequence was electronically published in the DNA database on 23 February 2000, does not hydrolyze ceftazidime. CTX-M-19 is a derivative of CTX-M-18 that can hydrolyze ceftazidime (19). If a single amino acid residue change occurs in the CTX-M-18 enzyme, this mutant (CTX-M-19) can acquire the capability to hydrolyze ceftazidime (12, 19). Accordingly, we believe that CTX-M-18 poses a higher risk when it is encountered in clinical isolates due to this potential ability to mutate into a ceftazidime-hydrolyzing enzyme.

ESBL-producing *Salmonella* and *Shigella* strains have been reported worldwide. Some 0.8 to 3.4% of clinical isolates of *Salmonella* recovered from 1997 to 1999 expressed the ESBL phenotype (29). Recently, investigators have also reported on CTX-M-3 (3), TEM-3 (1), and SHV-2a (13) ESBL-producing *Salmonella* isolates. However, to our knowledge, no ESBL-producing STEC or EHEC isolate has been described, until now.

In conclusion, this is the first report of an ESBL-producing STEC isolate. The possibility that this isolate may be an unsuspected reservoir of CTX-M-18 capable of spreading the plasmid carrying this ESBL to other bacteria might be worth

considering. It is necessary to extend antimicrobial resistance surveillance programs for *E. coli*, including STEC, not only to the clinical field but also to the agricultural field.

ACKNOWLEDGMENTS

This work was supported in part by two grants from the Ministry of Health, Labor and Welfare of Japan (Molecular Analyses of Drug-Resistant Bacteria and Establishment of Rapid Identification Methods [grant H15-Shinkou-9]) and by Toho University Project Research grant 15–4.

We are grateful to Kenneth S. Thomson, Creighton University School of Medicine, for useful advice and discussion.

REFERENCES

1. **AitMhand, R., A. Soukri, N. Moustaoui, H. Amarouch, N. ElMdaghri, D. Sirot, and M. Benbachir.** 2002. Plasmid-mediated TEM-3 extended-spectrum β-lactamase production in *Salmonella typhimurium* in Casablanca. J. Antimicrob. Chemother. **49:**169–172.
2. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. London Ser. B Biol. Sci. **289:**321–331.
3. **Baraniak, A., E. Sadowy, W. Hryniewicz, and M. Gniadkowski.** 2002. Two different extended-spectrum β-lactamases (ESBLs) in one of the first ESBL-producing *Salmonella* isolates in Poland. J. Clin. Microbiol. **40:**1095–1097.
4. **Bettelheim, K. A., and L. Beutin.** 2003. Rapid laboratory identification and characterization of verocytotoxigenic (Shiga toxin producing) *Escherichia coli* (VTEC/STEC). J. Appl. Microbiol. **95:**205–217.
5. **Beutin, L., G. Krause, S. Zimmermann, S. Kaulfuss, and K. Gleier.** 2004. Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol. **42:**1099–1108.
6. **Brett, K. N., V. Ramachandran, M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2003. stx_{1c} is the most common Shiga toxin 1 subtype among Shiga toxin-producing *Escherichia coli* isolates from sheep but not among isolates from cattle. J. Clin. Microbiol. **41:**926–936.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
8. **Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic.** 1999. Detection of Shiga-like toxin (stx_1 and stx_2), intimin (*eaeA*), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol. **65:**868–872.
9. **Fratamico, P. M., S. K. Sackitey, M. Wiedmann, and M. Y. Deng.** 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. **33:**2188–2191.
10. **Gannon, V. P., S. D'Souza, T. Graham, R. K. King, K. Rahn, and S. Read.** 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol. **35:**656–662.
11. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime- hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2269–2275.
12. **Kimura, S., M. Ishiguro, Y. Ishii, J. Alba, and K. Yamaguchi.** 2004. Role of a mutation at position 167 of CTX-M-19 in ceftazidime hydrolysis. Antimicrob. Agents Chemother. **48:**1454–1460.
13. **Mulvey, M. R., G. Soule, D. Boyd, W. Demczuk, and R. Ahmed.** 2003. Characterization of the first extended-spectrum β-lactamase-producing *Salmonella* isolate identified in Canada. J. Clin. Microbiol. **41:**460–462.
14. **Nagano, N., N. Shibata, Y. Saitou, Y. Nagano, and Y. Arakawa.** 2003. Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type β-lactamase. J. Clin. Microbiol. **41:**5530–5536.
15. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 6th ed., M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
16. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial susceptibility testing; 12th informational supplement, M100-S14. National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. **Osek, J.** 2003. Development of a multiplex PCR approach for the identification of Shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. J. Appl. Microbiol. **95:**1217–1225.
18. **Paton, J. C., and A. W. Paton.** 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. **11:**450–479.
19. **Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen, and P. Nordmann.** 2001. CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45:**3355–3361.
20. **Ritchie, J. M., C. M. Thorpe, A. B. Rogers, and M. K. Waldor.** 2003. Critical roles for stx_2, *eae*, and *tir* in enterohemorrhagic *Escherichia coli*-induced diarrhea and intestinal inflammation in infant rabbits. Infect. Immun. **71:**7129–7139.
21. **Russo, T. A.** 2001. Harrison's principles of internal medicine, 15th ed. McGraw-Hill Professional, New York, N.Y.
22. **Samadpour, M., M. Kubler, F. C. Buck, G. A. Depavia, E. Mazengia, J. Stewart, P. Yang, and D. Alfi.** 2002. Prevalence of Shiga toxin-producing *Escherichia coli* in ground beef and cattle feces from King County, Washington. J. Food Prot. **65:**1322–1325.
23. **Schroeder, C. M., J. Meng, S. Zhao, C. DebRoy, J. Torcolini, C. Zhao, P. F. McDermott, D. D. Wagner, R. D. Walker, and D. G. White.** 2002. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerg. Infect. Dis. **8:**1409–1414.
24. **Shiomi, M., M. Togawa, K. Fujita, and R. Murata.** 1999. Effect of early oral fluoroquinolones in hemorrhagic colitis due to *Escherichia coli* O157:H7. Pediatr. Int. **41:**228–232.
25. **Shiraki, Y., N. Shibata, Y. Doi, and Y. Arakawa.** 2004. *Escherichia coli* producing CTX-M-2 β-lactamase in cattle, Japan. Emerg. Infect. Dis. **10:**69–75.
26. **Tsuchizaki, N., J. Ishikawa, and K. Hotta.** 2000. Colony PCR for rapid detection of antibiotic resistance genes in MRSA and enterococci. Jpn. J. Antibiot. **53:**422–429.
27. **Winokur, P. L., R. Canton, J. M. Casellas, and N. Legakis.** 2001. Variations in the prevalence of strains expressing an extended-spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. Clin. Infect. Dis. **32**(Suppl. 2)**:**S94–S103.
28. **Yagi, T., H. Kurokawa, K. Senda, S. Ichiyama, H. Ito, S. Ohsuka, K. Shibayama, K. Shimokata, N. Kato, M. Ohta, and Y. Arakawa.** 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like β-lactamase genes. Antimicrob. Agents Chemother. **41:**2606–2611.
29. **Yagi, T., H. Kurokawa, N. Shibata, K. Shibayama, and Y. Arakawa.** 2000. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol. Lett. **184:**53–56.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2006, p. 1143–1147 Vol. 50, No. 4
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.4.1143–1147.2006

Organization of Tn*2610* Containing Two Transposition Modules

Akiko Takaya,[1] Masato Watanabe,[2]† and Tomoko Yamamoto[1]*

Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, 263-8522,[1] and Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, 174-8511,[2] Japan

Received 24 March 2005/Returned for modification 15 May 2005/Accepted 5 January 2006

Transposon Tn*2610*, found in a conjugative plasmid from an *Escherichia coli* isolate recovered at a hospital in Chiba, Japan, in 1975, was completely sequenced. Tn*2610* is 23,883 bp long and is bracketed by two transposition modules, a Tn*1721*-like module and a Tn*21*-derived module, which correspond, respectively, to the long inverted repeats IRa and IRb previously described for this transposon. Although both *tnpA* genes are intact, only that in the Tn*21*-derived module (IRb) functions in the transposition, while that in the Tn*1721*-derived module (IRa) cannot recognize the 38-bp imperfect repeat at the end of the IRb element. Both *tnpR* and *res* are present in IRa, while the *tnpR* gene of IRb is interrupted by the insertion of an IS*26* insertion element. The intervening region, between the *res* site of the Tn*1721* module and IS*26*, carries multiple integron-associated resistance genes within a Tn*21* backbone, including a region identical to that found in the genome of *Salmonella enterica* serovar Typhimurium DT104. These findings suggest that Tn*2610* originated from Tn*1721* and Tn*21*, with extensive recombination events with other elements which have resulted in a complex mosaic structure.

Tn*2610* is a multidrug resistance transposon that was originally identified in 1983 on a self-transmissible plasmid, pCS200, originating from an *Escherichia coli* strain isolated in 1975 at a hospital in Chiba, Japan (20). Tn*2610* is 24 kb long and is flanked by 3-kb inverted-repeat (IR) sequences. The intervening nonrepeated region was shown to include genes for resistance to ampicillin, streptomycin, and sulfonamide. Preliminary analysis revealed that Tn*2610* carries two copies of the transposition genes *tnpA* and *tnpR* and that these regions form a stable heteroduplex (19).

Several large transposons conferring resistance to more than one antibiotic have been identified. Among these, Tn*21*, Tn*1691*, Tn*2603*, and Tn*2424*, which are classified as class II transposons, seem to be evolutionarily related (11, 13, 18). On the basis of restriction maps and heteroduplex analyses, we originally proposed that the Tn*21*-related transposons had descended from an ancestral mercury resistance transposon, resembling Tn*2613*, by subsequent insertions of antibiotic resistance genes and/or insertion sequences (18). This hypothesis has been supported by sequence data from a large group of these transposons (11). Current knowledge on Tn*2610* suggests that it may also have evolved from an ancestral mercury resistance transposon via a series of recombination events resulting in a complex configuration.

To confirm this hypothesis, we determined the complete sequence of Tn*2610* and compared its structure to that of other known elements.

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, 263-8522, Japan. Phone: (81) 43-290-2928. Fax: (81) 43-290-2929. E-mail: tomoko-y@p.chiba-u.ac.jp.

† Present address: Astellas Pharma Inc., Tukuba, 300-2698, Japan.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *E. coli* DH5α (*supE44* Δ*lacU169* [φ80 *lacZ* ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), AB2463 (*recA thr leu thi lac gal ara xyl mtl pro his arg str tsx sup*), and P3478Rif, a rifampin-resistant mutant of P3478 (*thy polA*). The Tn*2610*-containing plasmid used in this study was pTKY170, formerly termed pMK1::Tn*2610*#4 (20). Subcloning for DNA sequencing was performed in pUC18 (17). Plasmids pTKY171 and pTKY172 are pAO3 derivatives carrying Tn*1722* and Tn*1722* with a kanamycin-resistant determinant, respectively. Plasmid pAO3 is a small derivative of plasmid ColE1 (21). Plasmid pTKY173 is a pTKY172 derivative defective in the *tnpA* gene. Plasmids pTKY174 and pTKY175 are pACYC184-based plasmids loaded with *tnpA* genes from Tn*2610* and Tn*1722*, respectively. Bacterial cells were routinely cultured at 37°C in Luria-Bertani (LB) medium or on LB agar. For selection with trimethoprim, Mueller-Hinton agar (Difco Laboratories) was used with 0.5% (vol/vol) lysed horse blood. Antibiotics were added at the following concentrations: ampicillin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 25 μg ml^{-1}; trimethoprim, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}.

Construction of plasmids. Plasmid pTKY171 (pAO3::Tn*1722*) was derived from plasmid pAO3::Tn*1721* by deletion of the DNA fragment between the SalI site at nucleotide (nt) 6946 in Tn*1721* (Fig. 1) and the BstEII site in pAO3. The pUC4K-derived kanamycin resistance determinant was inserted into the AvaI site at nt 991 in Tn*1722* (Fig. 1), resulting in pTKY172. Plasmid pTKY173 is an AvaI-generated deletion of pTKY172 which was cleaved at nt 2855 and nt 4562 in Tn*1722* and self-ligated. The DNA fragment between the EcoRI site at nt 13 and the BamHI site at nt 4157 in Tn*2610* from pTKY170 was cloned into BamHI-digested pACYC184, resulting in pTKY174. Plasmid pTKY175 was constructed by cloning of the DNA fragment between nt 13 and nt 5624 in Tn*1722* into EcoRI-digested pACYC184.

Determination of transposition proficiency. The mating-out assay was used to determine transposition frequency (19). The pACYC184-based plasmid loaded with a relevant *tnpA* gene was introduced into AB2463 containing R388, a conjugative plasmid devoid of transposable elements, and pTKY173. The resulting strain was used as a donor to mate with the recipient strain P3478Rif. Donor and recipient strains were mixed in a 1:5 ratio and passed through a Millipore filter, and then the filters were incubated on the agar plates at 37°C for 6 h. Transconjugants receiving R388::Tn*1722* were selected with kanamycin and rifampin. Transconjugants receiving R388 were selected with trimethoprim and rifampin. The transposition frequency was expressed as the ratio of the number of kanamycin-resistant transconjugants to the number of R388 transconjugants.

DNA isolation and restriction mapping. Plasmid DNA for restriction analysis and cloning was isolated by the alkaline lysis method (3). Restriction enzymes (TaKaRa Bio Inc., TOYOBO) were used in accordance with the manufacturer's