Table 3
Genotype and allele frequencies of *OAS-1* polymorphisms in SARS infected, uninfected, and controls without contact history

OAS-1	SARS infected ($n = 60$)	Uninfected ($n = 87$)	p value	Controls without contact ($n = 50$)
Exon 6				
Genotype				
AA	25 (41.7%)	53 (60.9%)	0.0215	27 (54.0%)
AG	28 (46.7%)	29 (33.3%)		17 (34.0%)
GG	7 (11.7%)	5 (5.7%)		6 (12.0%)
Allele				
A	0.65	0.76	0.0176	0.71
G	0.35	0.24		0.29
Exon 3				
Genotype				
AA	14 (23.3%)	37 (42.5%)	0.0163	17 (34.0%)
AG	33 (55.0%)	39 (44.8%)		26 (52.0%)
GG	13 (21.7%)	11 (12.6%)		7 (14%)
Allele				
A	0.51	0.65	0.0156	0.60
G	0.49	0.35		0.40

Table 4
Genotype and allele frequencies of *MxA* −88 G/T polymorphism in the subgroups of SARS cases

	SARS cases ($n = 44$)		p value
	Non-hypoxemic group ($n = 22$)	Hypoxemic group ($n = 22$)	
Genotype			
GG	8 (36.4%)	15 (68.2%)	0.0346
GT	10 (45.4%)	6 (27.3%)	
TT	4 (18.2%)	1 (4.5%)	
Allele			
G	0.59	0.82	0.0195
T	0.41	0.18	

In the presence of double-stranded RNA (dsRNA), OAS-1 catalyzes the 2′,5′-oligomers of adenosine in order to permit the binding and activation of a latent ribonuclease, RNase L, which cleaves cellular and viral RNAs [11,25]. *OAS-1* gene has two major transcripts that are generated by alternative splicing at the last two exons [23]. E16 (NM_002534) is a short transcript with 5 exons and is translated to p40 isoform. E18 (NM_016816) is a long transcript with 6 exons and is translated to p46 isoform. Another transcript 9-2 is generated using a different splice acceptor site that comes from E18 at exon 6 and is translated to 9-2 protein [26]. The 9-2 protein has a unique property due to the Bcl-2 homology domain 3 present in its unique carboxyl-terminal region. This is also distinctive in causing cellular apoptosis by binding to the anti-apoptotic proteins of the Bcl-2 family [26]. Therefore, OAS-1 has dual functions representing the synthesis of 2′,5′-oligomers of adenosine and the promotion of cellular apoptosis.

Knapp et al. [22] described how the GG genotype in exon 6 of *OAS-1* gene was more frequent in persistent HCV infection than in self-limiting infection. In our study, the G-allele was more frequently observed in SARS-CoV infected individuals than in the uninfected group. In both these studies, the G-allele was susceptible to virus infection. The A/G polymorphism in exon 6 is located downstream of the stop codon for E18 transcript meaning therefore that it is included in the 3′-untranslated region. However, it is located upstream of the stop codon for 9-2, and the A/G SNP results in amino acid substitution Arg397Gly of 9-2 protein, which is located near the Bcl-2 homology domain (amino acid positions 372–393). It will be an interesting aspect if this phenomenon occurs with any functional importance. We also analyzed the A/G polymorphism in exon 3 of *OAS-1* gene and found that there was strong linkage disequilibrium between the two SNPs. The A/G polymorphism in exon 3 causes amino acid substitution Ser162Gly in three isoforms, which is located near the dsRNA binding domain (amino acid positions 104–158) of OAS-1 [27]. We are unable at this point to determine which SNP is directly related to susceptibility to SARS or SARS-CoV infection. One can also consider that the other unidentified polymorphism of strong linkage disequilibrium with these SNPs may serve as the basis for any functional difference. Judging from the results obtained in this study, polymorphisms in *OAS-1* gene are likely to be involved in SARS-CoV infection or the development of SARS, at least in part, bearing in mind the fact that OAS-1 might have antiviral potential against SARS-CoV.

SARS-CoV is usually cultured in Vero E6 cell line [13–17,19], which cannot produce IFNs because it lacks *IFN* genes [28,29]. Recently, Cinatl et al. [30] infected permissive Caco-2 cells with SARS-CoV and analyzed the effects of SARS-CoV on cellular gene expression by high-density oligonucleotide arrays. They found that SARS-CoV infection of Caco-2 cells up-regulated IFN-inducible *OAS-2*, *OASL*, and *MxA* but not *PKR* genes. *OAS-2* and *OASL* are members of the human *OAS* gene family [25]. The role of OAS-1 as an inhibitor of SARS-CoV replication should be clarified to examine the hypothesis that Caco-2 cells permitted considerable infection with SARS-CoV because they did not induce *OAS-1*.

As regards the G/T polymorphism at position −88 in promoter region of *MxA* gene, GG genotype and G-allele were found to be more frequent in patients with an enhanced clinical progression, requiring oxygen therapy, although the number of cases was rather small. GG genotype was found more frequently in non-responders of IFN treatment in hepatitis C, and a luciferase reporter assay revealed that the *MxA* promoter sequence of G haplotype had lower promoter activity than that of T haplotype [31]. Recently, Arcas et al. [32] reported that GG genotype expressed lower amount of *MxA* mRNA than GT or TT genotype in IFN-treated peripheral

blood mononuclear cells in vitro. Spiegel et al. [15] reported that SARS-CoV replication was not affected in Vero E6 cells that were stably expressing MxA. They concluded that antiviral effect of IFN against SARS-CoV was not mediated by MxA. In our study, −88 SNP in *MxA* promoter was not related to disease susceptibility. Taking these observations together, MxA may not have a strong inhibitory effect on replication of SARS-CoV, but lower MxA expression may play a role in the worsening of SARS clinical progression.

If SARS re-emerges, IFN could be a promising candidate to treat SARS patients [12–19]. In the present study, the SNPs in *OAS-1* were associated with SARS-CoV infection or development of SARS, and the SNP in *MxA* was associated with the progression of SARS. It could be interesting to consider that they may also be related to the response of SARS patients to IFNs, and that SARS patients with AA genotype of the A/G SNP in exon 3 of *OAS-1* may respond to IFN treatment more effectively than those with AG or GG genotypes. During the course of our study, age was not a risk factor contributing to any worsening of SARS, probably because the majority of the patients consisted of relatively young medical staff members [6].

In conclusion, we showed that the polymorphisms in *OAS-1* gene were associated with SARS-CoV infection or development of SARS and that the polymorphism in *MxA* gene was also associated with hypoxemic status in SARS cases in Vietnam. These findings may lead to an understanding of IFN-induced antiviral response to SARS infection.

Acknowledgments

The authors thank Dr. Nguyen Le Hang, Ms. Pham Thi Phuong Thuy, and Ms. Nguyen Thi Thu Ha for their help in the management and coordination of this study in Vietnam. The authors also thank Kazuko Tanabe D.V.M. and Mr. John Crosskey for their critical reading of the manuscript and Dr. Goh Tanaka for his help in statistical analysis. This work was supported by a grant for International Health Cooperation Research (14C-9) and for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare in 2004.

References

[1] World Health Organization (2003), Consensus document on the epidemiology of severe acute respiratory syndrome (SARS), WHO/CDS/CSR/GAR/2003.11, Geneva.

[2] C.M. Booth, L.M. Matukas, G.A. Tomlinson, A.R. Rachlis, D.B. Rose, H.A. Dwosh, S.L. Walmsley, T. Mazzulli, M. Avendano, P. Derkach, I.E. Ephtimios, I. Kitai, B.D. Mederski, S.B. Shadowitz, W.L. Gold, L.A. Hawryluck, E. Rea, J.S. Chenkin, D.W. Cescon, S.M. Poutanen, A.S. Detsky, Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area, Jama 289 (2003) 2801–2809.

[3] J.W. Chan, C.K. Ng, Y.H. Chan, T.Y. Mok, S. Lee, S.Y. Chu, W.L. Law, M.P. Lee, P.C. Li, Short term outcome and risk factors for adverse clinical outcomes in adults with severe acute respiratory syndrome (SARS), Thorax 58 (2003) 686–689.

[4] M. Lin, H.K. Tseng, J.A. Trejaut, H.L. Lee, J.H. Loo, C.C. Chu, P.J. Chen, Y.W. Su, K.H. Lim, Z.U. Tsai, R.Y. Lin, R.S. Lin, C.H. Huang, Association of HLA class I with severe acute respiratory syndrome coronavirus infection, BMC Med. Genet. 4 (2003) 9.

[5] M.H. Ng, K.M. Lau, L. Li, S.H. Cheng, W.Y. Chan, P.K. Hui, B. Zee, C.B. Leung, J.J. Sung, Association of human-leukocyte-antigen class I (B*0703) and class II (DRB1*0301) genotypes with susceptibility and resistance to the development of severe acute respiratory syndrome, J. Infect. Dis. 190 (2004) 515–518.

[6] S. Itoyama, N. Keicho, T. Quy, N.C. Phi, H.T. Long, D. Ha le, V.V. Ban, J. Ohashi, M. Hijikata, I. Matsushita, A. Kawana, H. Yanai, T. Kirikae, T. Kuratsuji, T. Sasazuki, ACE1 polymorphism and progression of SARS, Biochem. Biophys. Res. Commun. 323 (2004) 1124–1129.

[7] J.S. Peiris, S.T. Lai, L.L. Poon, Y. Guan, L.Y. Yam, W. Lim, J. Nicholls, W.K. Yee, W.W. Yan, M.T. Cheung, V.C. Cheng, K.H. Chan, D.N. Tsang, R.W. Yung, T.K. Ng, K.Y. Yuen, Coronavirus as a possible cause of severe acute respiratory syndrome, Lancet 361 (2003) 1319–1325.

[8] T.G. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A.E. Ling, C.D. Humphrey, W.J. Shieh, J. Guarner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J.Y. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, A novel coronavirus associated with severe acute respiratory syndrome, N. Engl. J. Med. 348 (2003) 1953–1966.

[9] C. Drosten, S. Gunther, W. Preiser, S. van der Werf, H.R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R.A. Fouchier, A. Berger, A.M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J.C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H.D. Klenk, A.D. Osterhaus, H. Schmitz, H.W. Doerr, Identification of a novel coronavirus in patients with severe acute respiratory syndrome, N. Engl. J. Med. 348 (2003) 1967–1976.

[10] T. Kuiken, R.A. Fouchier, M. Schutten, G.F. Rimmelzwaan, G. van Amerongen, D. van Riel, J.D. Laman, T. de Jong, G. van Doornum, W. Lim, A.E. Ling, P.K. Chan, J.S. Tam, M.C. Zambon, R. Gopal, C. Drosten, S. van der Werf, N. Escriou, J.C. Manuguerra, K. Stohr, J.S. Peiris, A.D. Osterhaus, Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome, Lancet 362 (2003) 263–270.

[11] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (2001) 778–809.

[12] B.L. Haagmans, T. Kuiken, B.E. Martina, R.A. Fouchier, G.F. Rimmelzwaan, G. van Amerongen, D. van Riel, T. de Jong, S. Itamura, K.H. Chan, M. Tashiro, A.D. Osterhaus, Pegylated interferon-alpha protects type I pneumocytes against SARS coronavirus infection in macaques, Nat. Med. 10 (2004) 290–293.

[13] J. Cinatl, B. Morgenstern, G. Bauer, P. Chandra, H. Rabenau, H.W. Doerr, Treatment of SARS with human interferons, Lancet 362 (2003) 293–294.

[14] L.E. Hensley, L.E. Fritz, P.B. Jahrling, C.L. Karp, J.W. Huggins, T.W. Geisbert, Interferon-beta 1a and SARS coronavirus replication, Emerg. Infect. Dis. 10 (2004) 317–319.

[15] M. Spiegel, A. Pichlmair, E. Muhlberger, O. Haller, F. Weber, The antiviral effect of interferon-beta against SARS-coronavirus is not mediated by MxA protein, J. Clin. Virol. 30 (2004) 211–213.

[16] U. Stroher, A. DiCaro, Y. Li, J.E. Strong, F. Aoki, F. Plummer, S.M. Jones, H. Feldmann, Severe acute respiratory syndrome-

related coronavirus is inhibited by interferon-alpha, J. Infect. Dis. 189 (2004) 1164–1167.
[17] E.L. Tan, E.E. Ooi, C.Y. Lin, H.C. Tan, A.E. Ling, B. Lim, L.W. Stanton, Inhibition of SARS coronavirus infection in vitro with clinically approved antiviral drugs, Emerg. Infect. Dis. 10 (2004) 581–586.
[18] B. Zheng, M.L. He, K.L. Wong, C.T. Lum, L.L. Poon, Y. Peng, Y. Guan, M.C. Lin, H.F. Kung, Potent inhibition of SARS-associated coronavirus (SCOV) infection and replication by type I interferons (IFN-alpha/beta) but not by type II interferon (IFN-gamma), J. Interferon Cytokine Res. 24 (2004) 388–390.
[19] B. Sainz Jr., E.C. Mossel, C.J. Peters, R.F. Garry, Interferon-beta and interferon-gamma synergistically inhibit the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), Virology 329 (2004) 11–17.
[20] M. Hijikata, Y. Ohta, S. Mishiro, Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt −88) correlated with the response of hepatitis C patients to interferon, Intervirology 43 (2000) 124–127.
[21] F. Suzuki, Y. Arase, Y. Suzuki, A. Tsubota, N. Akuta, T. Hosaka, T. Someya, M. Kobayashi, S. Saitoh, K. Ikeda, M. Matsuda, K. Takagi, J. Satoh, H. Kumada, Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection, J. Viral Hepat. 11 (2004) 271–276.
[22] S. Knapp, L.J. Yee, A.J. Frodsham, B.J. Hennig, S. Hellier, L. Zhang, M. Wright, M. Chiaramonte, M. Graves, H.C. Thomas, A.V. Hill, M.R. Thursz, Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR, Genes Immun. 4 (2003) 411–419.
[23] P. Benech, Y. Mory, M. Revel, J. Chebath, Structure of two forms of the interferon-induced (2′–5′) oligo A synthetase of human cells based on cDNAs and gene sequences, Embo J. 4 (1985) 2249–2256.
[24] R.C. Lewontin, On measures of gametic disequilibrium, Genetics 120 (1988) 849–852.
[25] D. Rebouillat, A.G. Hovanessian, The human 2′,5′-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties, J. Interferon Cytokine Res. 19 (1999) 295–308.
[26] A. Ghosh, S.N. Sarkar, T.M. Rowe, G.C. Sen, A specific isozyme of 2′–5′ oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family, J. Biol. Chem. 276 (2001) 25447–25455.
[27] S.K. Ghosh, J. Kusari, S.K. Bandyopadhyay, H. Samanta, R. Kumar, G.C. Sen, Cloning, sequencing, and expression of two murine 2′–5′-oligoadenylate synthetases. Structure–function relationships, J. Biol. Chem. 266 (1991) 15293–15299.
[28] J.D. Mosca, P.M. Pitha, Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis, Mol. Cell. Biol. 6 (1986) 2279–2283.
[29] M.O. Diaz, S. Ziemin, M.M. Le Beau, P. Pitha, S.D. Smith, R.R. Chilcote, J.D. Rowley, Homozygous deletion of the alpha- and beta 1-interferon genes in human leukemia and derived cell lines, Proc. Natl. Acad. Sci. USA 85 (1988) 5259–5263.
[30] J. Cinatl Jr., G. Hoever, B. Morgenstern, W. Preiser, J.U. Vogel, W.K. Hofmann, G. Bauer, M. Michaelis, H.F. Rabenau, H.W. Doerr, Infection of cultured intestinal epithelial cells with severe acute respiratory syndrome coronavirus, Cell. Mol. Life Sci. 61 (2004) 2100–2112.
[31] M. Hijikata, S. Mishiro, C. Miyamoto, Y. Furuichi, M. Hashimoto, Y. Ohta, Genetic polymorphism of the MxA gene promoter and interferon responsiveness of hepatitis C patients: revisited by analyzing two SNP sites (−123 and −88) in vivo and in vitro, Intervirology 44 (2001) 379–382.
[32] N. Fernandez-Arcas, A. Blanco, M.J. Gaitan, M. Nyqvist, A. Alonso, A. Reyes-Engel, Differential transcriptional expression of the polymorphic myxovirus resistance protein A in response to interferon-alpha treatment, Pharmacogenetics 14 (2004) 189–193.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2005, p. 3734–3742 Vol. 49, No. 9
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.9.3734–3742.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Multidrug-Resistant *Pseudomonas aeruginosa* Strain That Caused an Outbreak in a Neurosurgery Ward and Its *aac(6′)-Iae* Gene Cassette Encoding a Novel Aminoglycoside Acetyltransferase

Jun-ichiro Sekiguchi,[1] Tsukasa Asagi,[2] Tohru Miyoshi-Akiyama,[1] Tomoko Fujino,[1] Intetsu Kobayashi,[3] Koji Morita,[4] Yoshihiro Kikuchi,[2] Tadatoshi Kuratsuji,[1,5] and Teruo Kirikae[1]*

Department of Infectious Diseases, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655,[1] National Hospital Organization, Sendai Medical Center, Miyagino 2-8-8, Miyagino, Sendai 938-8520,[2] Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., 3-30-1 Shimura, Itabashi, Tokyo 174-855,[3] Department of Microbiology, Kyorin University School of Health Sciences, 476 Miyashita, Hachioji, Tokyo 192-8508,[4] and National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535,[5] Japan

Received 23 February 2005/Returned for modification 15 April 2005/Accepted 15 June 2005

We characterized multidrug-resistant *Pseudomonas aeruginosa* strains isolated from patients involved in an outbreak of catheter-associated urinary tract infections that occurred in a neurosurgery ward of a hospital in Sendai, Japan. Pulsed-field gel electrophoresis of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates revealed that clonal expansion of a *P. aeruginosa* strain designated IMCJ2.S1 had occurred in the ward. This strain possessed broad-spectrum resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, sulfonamides, and chlorhexidine. Strain IMCJ2.S1 showed a level of resistance to some kinds of disinfectants similar to that of a control strain of *P. aeruginosa*, ATCC 27853. IMCJ2.S1 contained a novel class 1 integron, In113, in the chromosome but not on a plasmid. In113 contains an array of three gene cassettes of bla_{IMP-1}, a novel aminoglycoside resistance gene, and the *aadA1* gene. The aminoglycoside resistance gene, designated *aac(6′)-Iae*, encoded a 183-amino-acid protein that shared 57.1% identity with AAC(6′)-Iq. Recombinant AAC(6′)-Iae protein showed aminoglycoside 6′-*N*-acetyltransferase activity by thin-layer chromatography. *Escherichia coli* expressing exogenous *aac(6′)-Iae* showed resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin but not to arbekacin, gentamicins, or streptomycin. Alterations of *gyrA* and *parC* at the amino acid sequence level were detected in IMCJ2.S1, suggesting that such mutations confer the resistance to fluoroquinolones observed for this strain. These results indicate that *P. aeruginosa* IMCJ2.S1 has developed multidrug resistance by acquiring resistance determinants, including a novel member of the *aac(6′)-I* family and mutations in drug resistance genes.

Pseudomonas aeruginosa is intrinsically resistant to many antibiotics; however, it is sensitive to a limited number of drugs, including some β-lactams, such as ceftazidime and imipenem, and aminoglycosides, such as amikacin and tobramycin. However, recent studies have shown that several strains of *P. aeruginosa* that are resistant to these antibiotics have emerged and are becoming widespread (21, 28).

In Japan, the major mechanism of resistance to aminoglycosides is production of aminoglycoside-modifying enzymes (43). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides including amikacin, gentamicin, netilmicin, and tobramycin. The AAC(6′)-I type confers resistance to amikacin through acetylation of the drug, whereas the AAC(6′)-II type acetylates gentamicin. To date, several different genes, designated *aac(6′)-Ia* to *aac(6′)-Iad*, that encode the AAC(6′)-I enzymes have been cloned and characterized (42, 50). Genes encoding aminoglycoside-modifying enzymes are often located on integrons (15), sequences that can integrate gene cassettes through site-specific recombination (17), in both plasmid and genomic DNA (15). Class 1 integrons participate in multidrug resistance in *P. aeruginosa* (27, 28, 37). Class 1 integrons contain two conserved segments (CS) that flank the antibiotic resistance gene cassettes. The 5′-CS contains the *intI1* gene, which encodes integrase, the enzyme responsible for catalysis of site-specific recombination (8). The 3′-CS contains the *qacEΔ1* and *sul1* genes and an open reading frame (ORF), orf5 (13, 16).

We describe here the genotypic and phenotypic properties of a new multidrug-resistant *P. aeruginosa* strain that caused a nosocomial outbreak of infection at a hospital in Japan. The isolate carries a class 1 integron that contains an array of three gene cassettes, including one encoding a novel aminoglycoside acetyltransferase.

* Corresponding author. Mailing address: Department of Infectious Diseases, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. Phone: (81) 3 3202 7181, ext. 2838. Fax: (81) 3 3202 7364. E-mail:tkirikae@ri.imcj.go.jp.

MATERIALS AND METHODS

Bacterial strains. Seven clinical isolates of *P. aeruginosa*, including *P. aeruginosa* IMCJ2.S1, were obtained from seven patients with urinary tract infections in a neurosurgery ward of a hospital in Japan. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Manassas, Va). *Escherichia coli* strains DH5α (Takara Bio, Shiga, Japan) and BL21-AI (Invitrogen, Carlsbad, Calif.) were used as hosts for recombinant plasmids and for expression of *aac(6')-Iae*, respectively. The rifampin-resistant *P. aeruginosa* mutant ATCC 27853 RFP^r was used. *P. aeruginosa* GN17203 (51) was provided by S. Iyobe (Kitasato University, Sagamihara, Japan).

Antibiotics and disinfectants. The antibiotics amikacin, cefoxitin, and imipenem were from Banyu Pharmaceutical Co. (Tokyo, Japan). Arbekacin and dibekacin were from Meiji Seika Kaisha (Tokyo, Japan), aztreonam was from Eizai (Tokyo, Japan), cefotaxime was from Aventis Pharma (Tokyo, Japan), and cefpodoxime and ceftazidime were from Glaxo Smith Kline (Tokyo, Japan). Cefepime was from Bristol Pharmaceuticals (Tokyo, Japan); ciprofloxacin and levofloxacin were from Daiichi Pharmaceutical (Tokyo, Japan); gentamicin, isepamicin, netilmicin, and sisomicin were from Schering-Plough (Osaka, Japan); kanamycin A and B mixture, neomycin B and C mixture, and streptomycin were from Nacalai Tesque (Kyoto, Japan); and meropenem was from Sumitomo Pharmaceutical (Osaka, Japan). Tetracycline was from Lederle Japan Co. (Tokyo, Japan); piperacillin and piperacillin-tazobactam were from Tomiyama Pure Chemical Industries (Tokyo, Japan); moxalactam, tobramycin, and sulfamethoxazole-trimethoprim were from Shionogi and Co. (Osaka, Japan); and kanamycin A, polymyxin B, and silver sulfadiazine were from Sigma Chemical (St. Louis, Mo.). The disinfectants alkyldiaminoethylglycine hydrochloride and povidone iodine were from Yoshida Pharmaceutical Co. (Tokyo, Japan); benzalkonium chloride was from Wako Pure Chemical Industries (Osaka, Japan); and chlorhexidine gluconate was from Ishimaru Pharmaceutical (Osaka, Japan).

In vitro susceptibility to antibiotics and disinfectants. MICs of antibiotics, except polymyxin B and silver sulfadiazine, were determined by the microdilution method. The MICs of polymyxin B and silver sulfadiazine were determined by the agar dilution method according to the protocols recommended by the CLSI (formerly NCCLS), standard M7-A6 (33).

Bactericidal activities of disinfectants were evaluated by time- and dose-dependent killing studies in 96-well microplates. Briefly, 10^5 microorganisms were incubated at 35°C for 0.5 min to 60 min in 160 μl disinfectants diluted serially twofold. To neutralize the bactericidal activities of the disinfectants, a 10-μl aliquot of each suspension was transferred to 200 μl Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) containing 15% Tween 80 (Sigma), 1% soybean lecithin (Nacalai Tesque), and 0.5% sodium thiosulfate (Nacalai Tesque) and then cultured for 24 h. The minimum bactericidal concentrations (MBCs) of disinfectants were recorded relative to the duration of incubation with bacteria.

Transfer of drug resistance among bacteria. Transfer of the drug resistance from *P. aeruginosa* clinical isolates to a rifampin-resistant mutant of *P. aeruginosa*, ATCC 27853 RFP^r, was examined with the broth mating method (25). After mating, transconjugants were selected on Mueller-Hinton agar plates containing rifampin (200 μg/ml) and imipenem (16 μg/ml) or amikacin (20 μg/ml). Plasmid DNAs from the clinical isolates were purified either with a QIAprep kit (QIAGEN, Tokyo, Japan), by Kado and Liu's (24), or method by the method of Domenico et al. (11). With the QIAprep kit or Kado and Liu's method, the bacteria were lysed at different temperatures, 22°C for 5 min or 60°C for 70 min for each method.

PCR of class 1 integrons. To identify the presence of a class 1 integron and to determine the size of any inserted gene cassettes, PCR amplification was performed as described previously (29) with primers 5′-cs and 3′-cs, which are specific for 5′-CS and the 3′-CS of class 1 integrons, respectively, and an Expand High Fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany). To determine the content and order of genes in the integron, PCR amplification of the variable region of class 1 integrons was carried out with the primers listed in Table 1. All PCRs were performed with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). Genomic DNAs extracted as described by Sambrook and Russell (41) were used as templates. Amplification conditions were 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min or 5 min. PCR for amplicons longer than 1 kb was performed with 1.25 U of *Z-Taq* polymerase (Takara Bio) and 30 cycles of 95°C for 1 s and 68°C for 120 s according to the manufacturer's instructions.

PCR of QRDRs. The *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions (QRDRs) of *P. aeruginosa* were amplified by PCR with the primers listed in Table 1 according to methods described previously (1, 21, 26, 31). PCR products were sequenced with the same primers.

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems). Homology searches of nucleotide and deduced protein sequences were performed by FASTA and BLAST screens of the DDBJ, GenBank, and EMBL databases. Multiple-sequence alignments and searches for ORFs were performed with GENETYX-WIN software (Genetyx, Tokyo, Japan). The dendrogram for AACs was calculated with the CLUSTAL W Program (49).

Cloning of the *aac(6')-Iae* gene. The coding region of *aac(6')-Iae* (Fig. 1) was amplified by PCR with 2.5 U of *Ex Taq* DNA polymerase (Takara Bio) and primers aacS1-FC and aacS1-RC (Table 1). The PCR products were cloned into pCRT7/NT (Invitrogen) downstream of the region encoding a six-His tag. Then plasmid pAAC6, which contains *aac(6')-Iae*, or plasmid pREVAAC6, which contains *aac(6')-Iae* in the reverse direction, was transformed into *E. coli* DH5α cells by the $CaCl_2$ method (6). DNA sequences of these cloned fragments were verified by sequencing of both strands as described above.

Purification of recombinant AAC(6′)-Iae. *E. coli* BL21-AI harboring plasmid pAAC6 was grown to an A_{600} of 0.2 to 0.3 in LB medium containing 50 mg/liter ampicillin at 37°C. After addition of arabinose (final concentration, 0.02%) to induce expression of AAC(6′)-Iae, the *E. coli* strain was cultured for another 18 h at 25°C. The bacterial cells were collected, resuspended in 50 mM HEPES buffer (pH 7.5) containing 0.1% Triton X-100, and lysed by sonication on ice for 15 s 40 times and then for 20 s 100 times. After centrifugation to remove the debris, the solubilized protein was applied to an AKTA Prime (Amersham Biosciences, Piscataway, NJ) system equipped with a HiTrap Chelating HP column (Amersham Biosciences) loaded with Ni^{2+}. The column was washed with 20 mM Tris-HCl (pH 7.9) containing 60 mM imidazole and 0.5 M NaCl and was eluted with the same buffer containing 1 M imidazole. The eluted proteins were collected and dialyzed in 50 mM HEPES buffer (pH 7.5). The protein preparation yielded a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (data not shown).

Acetylation of aminoglycosides by recombinant ACC(6′)-Iae. Enzymatic acetylation of aminoglycosides was done as described previously (53). Recombinant AAC(6′) from actinomycete strain #8 was provided by J. Ishikawa (National Institute of Infectious Diseases, Tokyo, Japan). Various aminoglycosides were incubated with recombinant AAC(6′)-Iae or AAC(6′) as a positive control in the presence of acetyl coenzyme A, and the acetylated derivatives were detected by thin-layer chromatography. The reaction was carried out at 37°C for 30 min to 12 h.

Pulsed-field gel electrophoresis (PFGE). Genomic DNA from *P. aeruginosa* was prepared by the procedure of Grundmann et al. (14) and digested overnight with 10 U of SpeI, XbaI, or HpaI (Takara Bio). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, Calif.) at 6 V/cm for 20 h.

Southern hybridization. We performed Southern blotting to identify the location of In113. A 465-bp segment of *aac(6')-Iae* and a 362-bp segment of bla_{IMP-1} amplified by PCR were labeled with horseradish peroxidase and used as probes.

Nucleotide sequence accession number. The nucleotide sequence of In113 reported here has been deposited in the EMBL/GenBank/DDBJ databases and assigned accession number AB104852.

RESULTS

Epidemiologic analysis of a nosocomial outbreak of *P. aeruginosa*. From June 2002 to November 2002, a *P. aeruginosa* outbreak occurred in a neurosurgery ward of a 500-bed hospital in Japan. Three patients developed catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* in June 2002. Various measures for infection control were undertaken, but four patients subsequently developed similar catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* over the next 5 months. Seven *P. aeruginosa* isolates from these patients were analyzed by PFGE. The PFGE patterns of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates were identical, indicating that the isolates were all from monoclonal expansion of a single multidrug-resistant *P. aeruginosa* strain. This clone was named *P. aeruginosa* IMCJ2.S1. PFGE patterns of SpeI-, XbaI-, and

TABLE 1. PCR primers

Primer	Sequence[a] (5′→3′)	Expected size of amplicon (bp)	Position (nt)[b]	Reference or GenBank accession no.
5′-cs	GGCATCCAAGCAGCAAG			29
3′-cs	AAGCAGACTTGACCTGA			29
int1-F	TGCGTGTAAATCATCGTCGT	838	Downstream of *intI1*	AF071413
int1-R	CGAAGTCGAGGCATTTCTGT		177–196 in *intI1*	AF071413
IMP-F[c]	DTTYCTAAACAYGGYTTGGT	362	145–164 in bla_{IMP-1}	AB070224
IMP-R[c]	YTTTYAGGYARCCAAACYACT		486–506 in bla_{IMP-1}	AB070224
aacS1-F	CGCAAGCTGCAGAAATTCTAT	465	47–67 in *aac(6′)-Iae*	This study
aacS1-R	TCCCATTTGCATTAGGAATCA		491–511 in *aac(6′)-Iae*	This study
aadA1-F	TGATTTGCTGGTTACGGTGA	451	144–163 in *aadA1*	AF071413
aadA1-R	TACTGCGCTGTACCAAATGC		575–594 in *aadA1*	AF071413
qacEdelta-F	TGAAAGGCTGGCTTTTTCTT	286	2–21 in *qacEΔ1*	AF071413
qacEdelta-R	GCAATTATGAGCCCCATACC		268–287 in *qacEΔ1*	AF071413
sul-F	TCACCGAGGACTCCTTCTTC	759	29–48 in *sul1*	AF071413
sul-R	GGGTTTCCGAGAAGGTGATT		768–787 in *sul1*	AF071413
int1imp1-F	AGCACCTTGCCGTAGAAGAA	695	262–281 in *intI1*	AJ640197
int1imp1-R	TTTTATAGCCACGCTCCACA		243–262 in bla_{IMP-1}	AJ640197
imp1aacS1-F	AAAGGCAGCATTTCCTCTCA	737	265–284 in bla_{IMP-1}	This study
imp1aacS1-R	GACGGCCAAGAATCGAAAT		89–107 in *aac(6′)-Iae*	This study
aacS1aadA1-F	ATTGTGTGGTTGGGTTGGAT	691	186–205 in *aac(6′)-Iae*	This study
aacS1aadA1-R	GGAGAATCTCGCTCTCTCCA		231–259 in *aadA1*	This study
aadA1qacEd-F	TGATTTGCTGGTTACGGTGA	873	144–163 in *aadA1*	AF071413
aadA1qacEd-R	ATGCGGATGTTGCGATTACT		42–61 in *qacEΔ1*	AF071413
qacEdsul-F	TCGGTGTTGCTTATGCAGTC	306	167–186 in *qacEΔ1*	AF071413
qacEdsul-R	ACATCCACGACGTCTGATCC		112–131 in *sul1*	AF071413
int-R	TGCGTGTAAATCATCGTCGT	3,172	Downstream of *intI1*	AF071413
sul-R	GGGTTTCCGAGAAGGTGATT		768–787 in *sul1*	AF071413
sul-F	TCACCGAGGACTCCTTCTTC	6,474	29–48 in *sul1*	AF071413
tniB-R	ATCATCGACCTGTCCCACCT		16–35 in *tniBΔ1*	AF071413
tniB-F	CAGAGCCAGTTGCTCCATTT	1,749	395–414 in *tniBΔ1*	AF071413
tniA-R	CTTTCACCGCGAAGTCACTC		384–403 in *tniA*	AF071413
GyrA1	TTATGCCATGAGCGAGCTGGGCAACGACT	366	147–176 in *gyrA*	26
GyrA2	AACCGTTGACCAGCAGGTTGGGAATCTT		484–512 in *gyrA*	26
GyrB1	GCGCGTGAGATGACCCGCCGT	390	1162–1182 in *gyrB*	31
GyrB2	CTGGCGGTAGAAGAAGGTCAT		1531–1551 in *gyrB*	31
PARC1	ATGAGCGAACTGGGGCTGGAT	210	166–187 in *parC*	21
PARC2	ATGGCGGCGAAGGACTTGGGA		354–375 in *parC*	21
ParE1	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	1223–1250 in *parE*	1
ParE2	TCGAGGGCGTAGTAGATGTCCTTGCCGA		1787–1814 in *parE*	1
aacS1-FC	ATGAAATACAACATTGTTAATATTA	552	1–25 in *aac(6′)-Iae*	This study
aacS1-RC	TTACATTATATTTTTCCACATTAAT		528–552 in *aac(6′)-Iae*	This study

[a] D stands for adenine, thymine, or guanine; R stands for adenine or guanine; Y stands for cytosine or thymine.
[b] Nucleotides are numbered according to deposited sequences.
[c] Primer designed to amplify bla_{IMP-1} (accession no. AB070224) or homologous genes, including bla_{IMP-2} (AJ243491), bla_{IMP-3} (AB010417), bla_{IMP-4} (AF445082), bla_{IMP-5} (AF290912), bla_{IMP-6} (AB040994), bla_{IMP-7} (AF416736), bla_{IMP-8} (AF322577), bla_{IMP-9} (AY033653), bla_{IMP-10} (AB074434), and bla_{IMP-11} (AB074437).

HpaI-digested genomic DNAs from IMCJ2.S1 are shown in Fig. 2A.

Susceptibility of *P. aeruginosa* IMCJ2.S1 to antibiotics and disinfectants. The MICs of various antibiotics, including potent active β-lactams, against IMCJ2.S1 were compared with those against a reference strain, *P. aeruginosa* ATCC 27853 (Table 2). IMCJ2.S1 was resistant to all antibiotics tested except for arbekacin and polymyxin B. Strain ATCC 27853 was sensitive to all of the antibiotics tested except cefoxitin, flomoxef, and kanamycin. Thus, IMCJ2.S1 was classified as a multidrug-resistant strain of *P. aeruginosa*.

To test whether IMCJ2.S1 showed increased resistance to disinfectants, the MBCs of four disinfectants, povidone iodine, alkyldiaminoethylglycine hydrochloride, benzalkonium chloride, and chlorhexidine gluconate, were determined for both IMCJ2.S1 and ATCC 27853. Both strains were resistant to chlorhexidine gluconate but sensitive to povidone iodine (MBC, <0.001% [wt/vol]), alkyldiaminoethylglycine hydrochloride (MBC, <0.001% [wt/vol]), and benzalkonium choride (MBC, <0.005% [wt/vol]). The MBC patterns of these strains were identical. These results indicate that the sensitivity of IMCJ2.S1 to disinfectants is not different from that of the *P. aeruginosa* reference strain.

Detection of an integron in *P. aeruginosa* IMCJ2.S1. To determine if strain IMCJ2.S1 carried a class 1 integron, PCR analysis specific for class 1 integrons was performed (29). Strain IMCJ2.S1 yielded a 2.5-kbp PCR product, whereas *E. coli* CSH2 harboring plasmid NR1 (32), which carries In2 (30), yielded a 1.0-kbp PCR product. *P. aeruginosa* ATCC 27853 did not yield PCR products. These results suggest that strain IMCJ2.S1 and *E. coli* CSH2 each carry a class 1 integron and that this integron contains additional sequences that are not present in In2.

The class 1 integron frequently contains the *tniB* and *tniA* genes downstream of the 3′-CS (13, 16). To confirm the presence of a class 1 integron in IMCJ2.S1 and to elucidate the

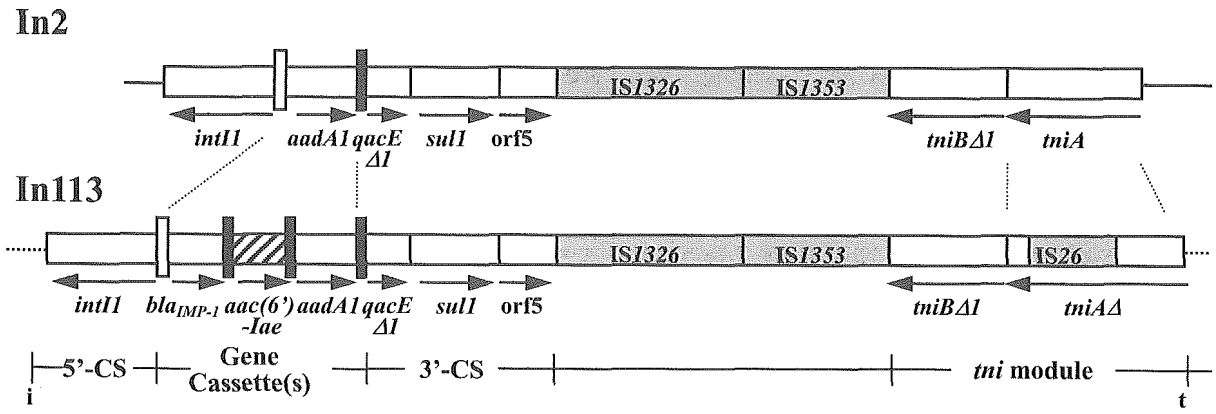

FIG. 1. Structure of In2 (GenBank accession no. AF071413) and In113. Gene cassettes are represented as open boxes with an adjacent vertical bar (59-be), shown as heavy solid vertical bars. The novel ORF found in In113 is shown as a hatched box. Genes are indicated by horizontal arrows. IS are represented as gray boxes and are labeled. The sites of the 5′-CS, gene cassettes, 3′-CS, and *tni* module are indicated just below the construct. IRi and IRt are shown as vertical lines labeled i and t, respectively, and the *attI1* sites are shown as open vertical bars toward the left of the constructs.

structure downstream of the 3′-CS, we performed PCR specific for *intI1*, *qacEΔ1*, *sul1*, and their spanning or marginal regions. PCRs yielded the expected products (Table 1), with the exception of a 4.7-kbp fragment after amplification with int-R and sul-R and a 2.5-kbp fragment after amplification with tniB-F and tniA-R. These data show that IMCJ2.S1 carries a class 1 integron and that this integron contains *intI1-sul1* in a 4.7-kbp region, *sul1-tniB* in a 6.5-kbp region, and *tniB-tniA* in a 2.5-kbp region (Fig. 1).

Identical results were obtained for the other six isolates from the outbreak.

Structure of the class 1 integron found in *P. aeruginosa* IMCJ2.S1. We analyzed the sequences of the PCR products to determine the structure of the class 1 integron of IMCJ2.S1. The 5′-CS contained *intI1*, the *attI1* recombination site with a 7-bp core site sequence of GTTAGAA (45), and the TGGACA (−35) and TAAACT (−10) hexamers separated by 17 bp, which is characteristic of the Pc promoter (7, 45). Although TTGTTA (−35) and TACAGT (−10) hexamers separated by 14 bp were present again downstream of the Pc promoter, this region is not likely to act as the P2 promoter, because there is no GGG sequence (7, 45).

Between the 5′-CS and 3′-CS, there were three gene cassettes (Fig. 1). The 880-nucleotide (nt) cassette contained the metallo-β-lactamase gene bla_{IMP-1} (35) and a 127-nt 59-base element (59-be) site, a site for site-specific cointegration events (Fig. 3), and this cassette was identical to one described previously (2, 35). The 647-nt cassette contained an ORF and a 68-nt 59-be site (Fig. 3). The sequence of this 647-nt cassette was not found in any database, and therefore, we named this integron In113 (Fig. 1). The ORF in the 647-nt cassette encoded a 183-amino-acid (aa) product that was 55.2% identical to a 6′-*N*-aminoglycoside acetyltransferase, AAC(6′)-Ia (48), and 57.1% identical to AAC(6′)-Iq of *Klebsiella pneumoniae* (4). We named the predicted protein AAC(6′)-Iae according to the standard nomenclature (42).

AAC(6′)-Iae was relatively similar to a subfamily of AAC(6′)-I enzymes that includes AAC(6′)-Ia (48), AAC(6′)-Iq (4), and AAC(6′)-Im (19) [which is not the AAC(6′)-Im reported by Chow et al. (5) and has also been

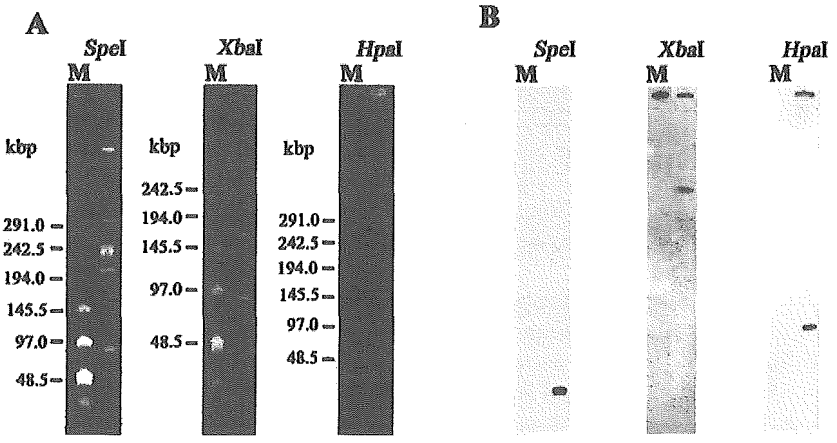

FIG. 2. (A) PFGE of SpeI-, XbaI-, and HpaI-digested genomic DNA from multidrug-resistant *P. aeruginosa* IMCJ2.S1. (B) Southern blotting of the same gels with an *aac(6′)-Iae* probe. Lanes M, HindIII-digested λ phage DNA as a size marker.

TABLE 2. In vitro susceptibilities of *P. aeruginosa* IMCJ2.S1 and *P. aeruginosa* ATCC 27853 to various antimicrobial agents

Antibiotic	MIC (μg/ml) for:	
	P. aeruginosa IMCJ2.S1	*P. aeruginosa* ATCC 27853
Piperacillin	>128	<4
Piperacillin-tazobactam	64	4
Cefotaxime	>128	8
Ceftazidime	>128	<1
Cefepime	>64	2
Cefoxitin	>64	>64
Flomoxef	>128	>128
Moxalactam	>128	16
Imipenem	128	4
Meropenem	128	1
Aztreonam	128	2
Amikacin	128	2
Arbekacin	2	<0.5
Dibekacin	>128	<0.5
Gentamicin	16	<1
Isepamicin	128	<4
Kanamycin	>128	>128
Netilmicin	>128	<0.5
Sisomicin	>128	<0.5
Streptomycin	>64	<4
Tobramycin	64	<0.5
Tetracycline	32	16
Sulfamethoxazole-trimethoprim	128	32
Levofloxacin	64	<0.5
Ciprofloxacin	32	<0.5
Polymyxin B	2	2
Silver sulfadiazine	64	64

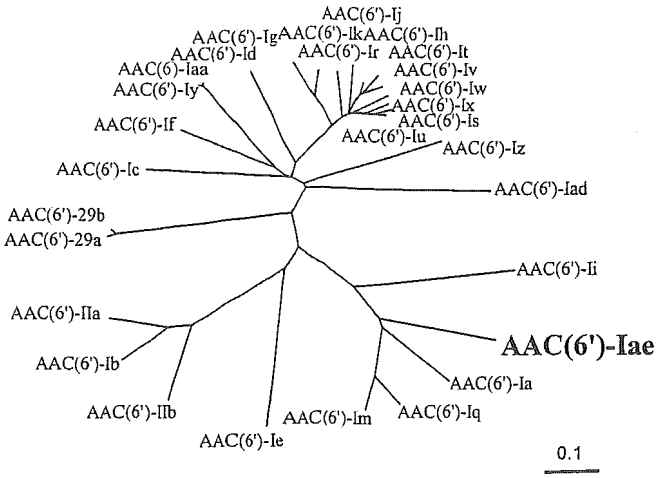

FIG. 4. Dendrogram of aminoglycoside 6′-*N*-acetyltransferases for comparison with AAC(6′)-Iae. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for AAC proteins. EMBL/GenBank/DDBJ accession numbers of AAC proteins are as follows: AAC(6′)-Ia, M18967-1; AAC(6′)-Ib, M23634; AAC(6′)-Ic, M94066; AAC(6′)-Id, X12618; AAC(6′)-Ie, M13771; AAC(6′)-If, X55353; AAC(6′)-Ig, L09246; AAC(6′)-Ih, L29044; AAC(6′)-Ii, L12710-1; AAC(6′)-Ij, L29045; AAC(6′)-Ik, L29510; AAC(6′)-Il, Z54241 and U13880; AAC(6′)-Im, Z54241-2; AAC(6′)-Iq, AF047556-1; AAC(6′)-Ir, AF031326; AAC(6′)-Is, AF031327; AAC(6′)-It, AF031328; AAC(6′)-Iu, AF031329; AAC(6′)-Iv, AF031330; AAC(6′)-Iw, AF031331; AAC(6′)-Ix, AF031332; AAC(6′)-Iy, AF144880; AAC(6′)-Iz, AF140221; AAC(6′)-Iaa, NC_003197; AAC(6′)-Iad, AB119105; AAC(6′)-IIa, M29695; AAC(6′)-IIb, L06163; AAC(6′)-29a, AF263519; AAC(6′)-29b, AF263519.

referred to as AAC(6′)-Ip, by Centrón and Roy (4)] (61.7% identity in a 149-aa overlap) and to AAC(6′)-Ii (9) (40.3% identity in a 166-aa overlap) (Fig. 4). On the basis of the work of Neuwald and Landsman (34), four motifs in the amino acid sequences of the subfamily proteins belonging to AAC(6′)-Iae were designated motifs C, D, A, and B (Fig. 5). Comparison of amino acid sequences of members of the AAC(6′)-I subfamily with that of AAC(6′)-Iae revealed that motifs C, D, A, and B, which are found in most GCN5-related *N*-acetyltransferases (GNATs) (12, 34), were conserved in AAC(6′)-Iae (Fig. 5). A large motif at the C terminus, motif B (12), was 63.3% identical between AAC(6′)-Im (19) and AAC(6′)-Iae. The third cassette was 856 nt long and contained the aminoglycoside 3″-adenyltransferase gene *aadA1* (18, 22) and a 60-nt 59-be site (Fig. 3). This cassette was similar to one reported previously (30, 36) except for a silent C-to-T substitution at nt 135.

The 3′-CS included *qacEΔ1* (39), *sul* (47), and orf5 (30, 37). There were three inserted sequences (IS), IS*1326* (3), IS*1353* (3), and IS*26* (38), in the region downstream of the 3′-CS (Fig. 1). IS*26* is known to be inserted into the *tniA* coding region of the *tni* transposition module (30).

Drug resistance mediated by the AAC(6′)-Iae enzyme. To examine the role of AAC(6′)-Iae in aminoglycoside resistance, a recombinant plasmid, pAAC6, carrying *aac(6′)-Iae* from strain IMCJ2.S1 was transformed into *E. coli* DH5α. *E. coli* harboring pAAC6 showed significantly lower susceptibility to

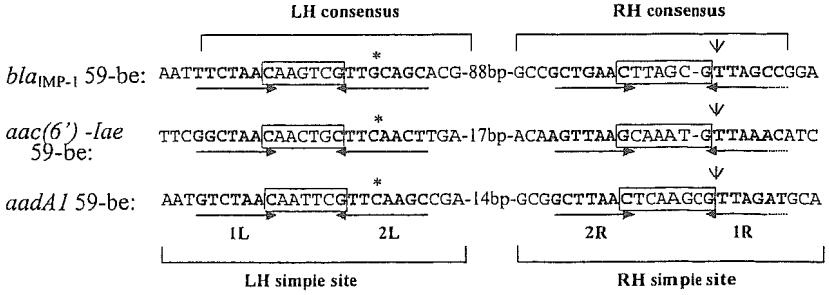

FIG. 3. Structures of 59-be of In113. Seven-base-pair putative core sites in the left-hand (LH) and right-hand (RH) consensus sequences were designated 1L and 2L and 2R and 1R, respectively. The putative recombination event occurs between the G and the first T in the 1R core site and is indicated by vertical arrows (see reference 45). The relative orientations of 1L, 2L, 2R, and 1R are indicated by arrows under the sequence. An extra base in 2L is marked with an asterisk. Inverted repeats are underscored with arrows.

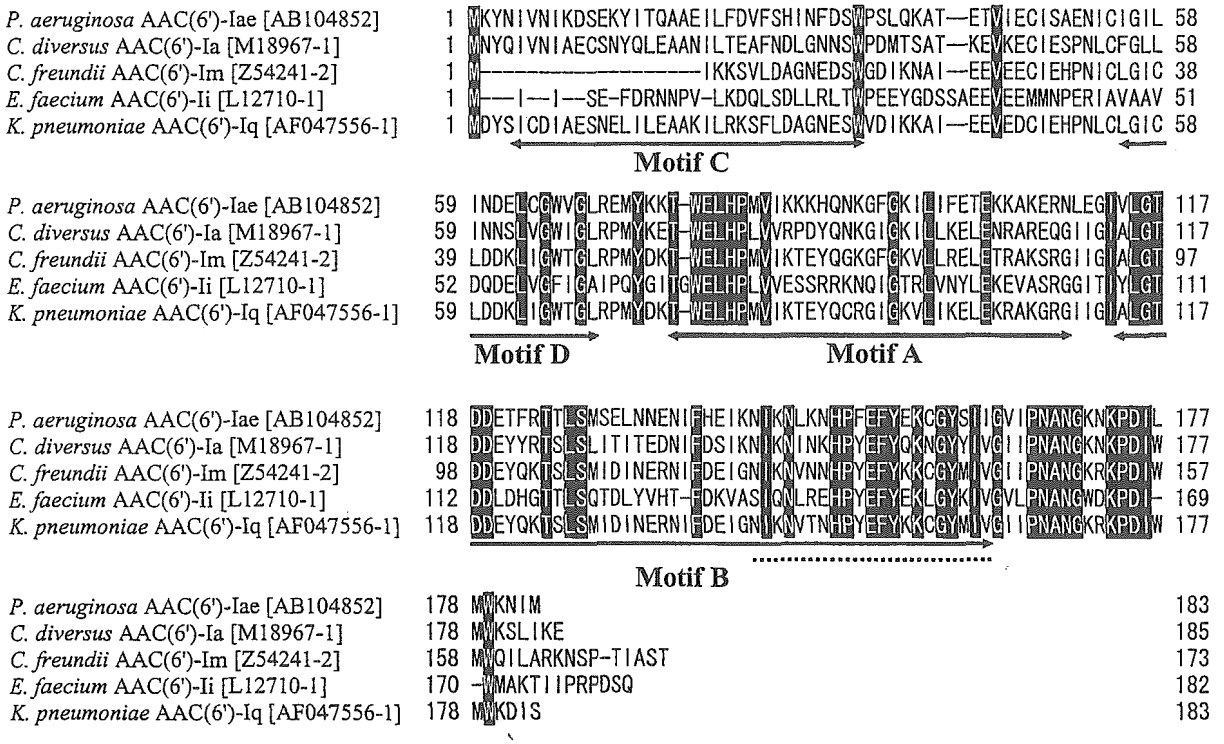

FIG. 5. Alignment of the AAC(6′)-Iae amino acid sequence with those of four members of the AAC(6′)-I subfamily. Identical residues are marked with black boxes. Four motifs, including the highly conserved motif B, are underlined. A conserved region of 21 amino acids, described by Shmara et al. (44), is indicated by a dotted line. GenBank accession numbers are given in brackets to the right of AAC names. *C. diversus*, *Citrobacter diversus*; *C. freundii*, *Citrobacter freundii*.

amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin than the parent strain and the negative control. MICs for other aminoglycosides, including arbekacin, gentamicin, and streptomycin, were unchanged (Table 3). These results indicate that *aac(6′)-Iae* is involved in aminoglycoside resistance.

To examine potential acetylase activity of AAC(6′)-Iae, we assessed the purified recombinant AAC(6′)-Iae against aminoglycosides by thin-layer chromatography (53). As shown in Fig. 6, kanamycin, amikacin, tobramycin, netilmicin, sisomicin, isepamicin, arbekacin, neomycin, and gentamicin were acetylated by AAC(6′)-Iae and AAC(6′). Acetylation by AAC(6′)-Iae was complete for all of these aminoglycosides except gentamicin, which showed incomplete acetylation. These aminoglycosides all have 6′-NH_2. The present results, therefore, suggest that AAC(6′)-Iae is a functional acetyltransferase that modifies the 6′-NH_2 position of aminoglycosides.

Location of In113. Clinical isolates of *P. aeruginosa* frequently possess the R plasmid, which carries a class 1 integron. Therefore, we screened our seven *P. aeruginosa* clinical isolates for the presence of this plasmid. *P. aeruginosa* GN17203 was used as a positive control for bla_{IMP-1}, since it has been shown to harbor pMS350, which contains a bla_{IMP-1} gene. Genomic DNA from IMCJ2.S1 was used as a control for *aac(6′)-Iae* and bla_{IMP-1}.

The extracts from the seven clinical isolates and *P. aeruginosa* GN17203 were separated by agarose gel electrophoresis, and Southern blotting with *aac(6′)-Iae* or bla_{IMP-1} as a probe was performed. A plasmid that contained bla_{IMP-1} but not *aac(6′)-Iae* was detected in *P. aeruginosa* GN17203. Despite

TABLE 3. Aminoglycoside resistance patterns of *E. coli* DH5α alone or harboring plasmids with or without *aac(6′)-Iae*

Strain	MIC (μg/ml)[a] of:										
	AMK	ABK	DIB	GEN	ISE	KAN	NEO	NET	SIS	STR	TOB
E. coli DH5α(pAAC6)[b]	8	0.5	32	0.25	8	64	4	32	16	4	8
E. coli DH5α(pREVAAC6)[c]	0.5	0.5	0.5	0.25	0.25	1	2	0.25	0.25	4	0.5
E. coli DH5α(pCRT7/NT)[d]	0.5	0.25	0.5	0.25	0.25	1	2	0.25	0.25	4	0.25
E. coli DH5α	0.5	0.5	0.5	0.25	0.25	1	2	0.25	0.25	4	0.25

[a] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEN, gentamicin; ISE, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; STR, streptomycin; TOB, tobramycin.
[b] Recombinant plasmid constructed by cloning *aac(6′)-Iae* into pCRT7/NT.
[c] Recombinant plasmid constructed by insertion of DNA fragment with reverse sequence of *aac(6′)-Iae* into pCRT7/NT.
[d] Cloning vector (ABPC).

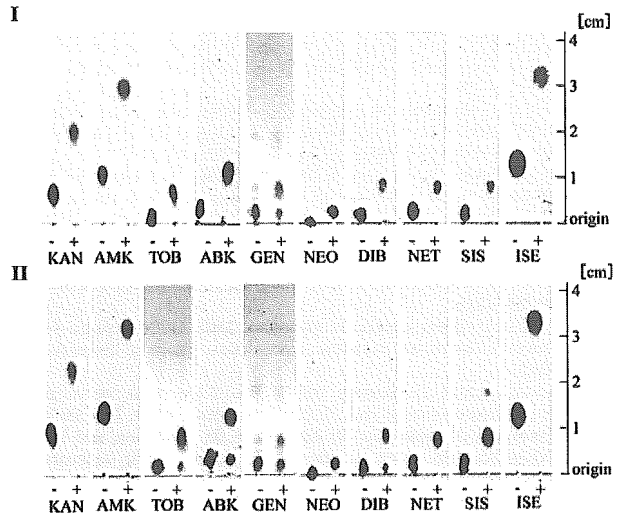

FIG. 6. Thin-layer chromatogram of aminoglycosides incubated with AAC(6′)-Iae protein (I) or with AAC(6′) from *Streptomyces lividans* TK21 as a control (II) (53) in the presence (+) or absence (−) of acetyl coenzyme A. KAN, kanamycin; AMK, amikacin; TOB, tobramycin; ABK, arbekacin; GEN, gentamicin; NEO, neomycin; DIB, dibekacin; NET, netilmicin; SIS, sisomicin; ISE, isepamicin.

repeated attempts (three times per procedure), we did not detect this plasmid by ethidium bromide staining or Southern blotting in any of the clinical isolates (data not shown). In contrast, Southern hybridization of SpeI-, XbaI-, and HpaI-digested genomic DNAs of the seven clinical isolates revealed 50-kb, 250-kb, and 60-kb *aac(6′)-Iae*-positive fragments, respectively (Fig. 2). These fragments were also positive for bla_{IMP-1} (data not shown). To examine whether the drug-resistant phenotype of *P. aeruginosa* IMCJ2.S1 can be transferred by conjugation, IMCJ2.S1 was incubated with *P. aeruginosa* ATCC 27853 RFP^r. Carbapenem resistance was transferred from *P. aeruginosa* GN17203 to *P. aeruginosa* ATCC 27853 RFP^r, consistent with the results reported by Watanabe et al. (51). In contrast, resistance to amikacin or carbapenem was not transferred from IMCJ2.S1 to ATCC 27853 RFP^r. These results suggest that In113 is located in the chromosome, and not on a plasmid, of *P. aeruginosa* IMCJ2.S1.

Resistance of IMCJ2.S1 to fluoroquinolones. IMCJ2.S1 was highly resistant to fluoroquinolones (Table 2). This resistance is typically associated with mutations in the QRDR within *gyrA*, *gyrB*, *parC*, and *parE*, which encode DNA gyrase or topoisomerase IV in *P. aeruginosa* (1, 21, 26, 31). Therefore, we screened IMCJ2.S1 mutations within the QRDR. Compared to the *gyrA* sequence of strain PAO1 (46), the *gyrA* sequence of IMCJ2.S1 contained an ACC-to-ATC mutation in codon 83 that causes a Thr-to-Ile change in the A subunit of DNA gyrase. IMCJ2.S1 also had a TCG-to-TTG mutation in codon 87 of *parC* that causes a Ser-to-Leu substitution in the C subunit of topoisomerase IV. IMCJ2.S1 had four mutations in *gyrB*: CGC to CGT in codon 396, AAA to AAG in codon 408, GAA to GAG in codon 484, and TTG to CTG in codon 513. There were four mutations in *parE*: GAA to GAG in codon 448, GGT to GGC in codon 472, AGT to AGC in codon 474, and GCC to GCT in codon 477. These mutations in *gyrB* and *parE* did not lead to amino acid changes in the proteins encoded (1, 31). Identical results were obtained with the other six clinical isolates. Together, these results indicate that IMCJ2.S1 contains mutations in *gyrA* and *parC* that are associated with its fluoroquinolone resistance.

DISCUSSION

A variety of aminoglycoside 6′-*N*-acetyltransferases have been described (Fig. 4) and classified into three subgroups (42, 50). Recently, a new enzyme, AAC(6′)-Iad, which is a member of the largest subfamily, was isolated from an *Acinetobacter* genospecies 3 strain in Japan (10). In the present study, we identified AAC(6′)-Iae, which shows considerable phylogenetic distance from members of the largest subfamily, which includes AAC(6′)-Iad and its divergents (Fig. 4). AAC(6′)-Iae belongs to the subfamily comprising AAC(6′)-Ia, -Ii, -Im, and -Iq (4, 9, 19, 48). There was only a low level of homology between the 59-be site of *aac(6′)-Iae* and those of the genes encoding other members of the *aac(6′)-I* family. Furthermore, *aac(6′)-Iae* has a low G+C content (26.8%) (data not shown), whereas the average G+C content of the *P. aeruginosa* PAO1 genome is 66.6% (46). Therefore, *aac(6′)-Iae* may be derived from an environmental species with an intrinsically low G+C content.

AAC(6′)-Iae from *P. aeruginosa* strain IMCJ2.S1, which was responsible for an outbreak of catheter-associated urinary tract infections, acetylated all of the aminoglycosides with 6′-NH_2, and acetylation of arbekacin and neomycin appeared to be complete (Fig. 6I). However, *E. coli* DH5α(pAAC6), expressing exogenous AAC(6′)-Iae, was sensitive to arbekacin and did not show reduced susceptibility to neomycin. Arbekacin and neomycin were shown to retain their antibiotic effects even after they were acetylated by AAC(6′) from an arbekacin-resistant actinomycete strain at the 6′ positions (53). *Enterococcus faecium* producing AAC(6′)-Ii was susceptible to neomycin even though AAC(6′)-Ii acetylated neomycin (52). These results suggest that acetylation of arbekacin and neomycin at 6′ positions does not affect the antimicrobial activities of these drugs. We cannot exclude the possibility that the antimicrobial activity observed after treatment with AAC(6′)-Iae is due to residual arbekacin or neomycin that was not acetylated.

E. coli DH5α expressing AAC(6′)-Iae was sensitive to gentamicin (Table 3), although AAC(6′)-Iae showed only partial acetylation of gentamicin (Fig. 6I). The sensitivity of these bacteria to gentamicin appears to be due to incomplete acetylation of gentamicin, which was observed with AAC(6′) from an arbekacin-resistant actinomycete strain (53)(Fig.6II). Commercially available gentamicin is a mixture of a number of derivatives of gentamicin, such as gentamicin C_1, C_{1a}, C_2, and C_{2b}, that have modifications of position 6′. Gentamicin C_1 and C_{2b} carry a methyl group on N-6′ and are refractory to AAC(6′)-I enzymes (42, 50). We cannot exclude the possibility that acetylated gentamicin components, which are more susceptible to AAC(6′)-I enzymes, retain antibiotic activity.

In the present study, we identified In113, a class 1 integron that contains a novel aminoglycoside resistance gene, *aac(6′)-Iae*. Several classes of integrons have been categorized on the basis of the structure of integrase (15, 40). The most common integrons in *P. aeruginosa* are those of class 1 (27, 28, 37).

Because their structures are very similar to each other, the direct origin of In113 could be from In2 (30), which was originally isolated from *Shigella flexneri* in Japan in the late 1950s (32) (Fig. 1).

IMCJ2.S1 was resistant to all antibiotics tested except arbekacin and polymyxin B (Table 2). However, the presence of In113 and the mutations in *gyrA* and *parC* of the QRDR are not sufficient to explain the multidrug resistance of this strain. Alterations of *gyrA* and *parC* are known to contribute to fluoroquinolone resistance (1, 21, 26, 31). The bla_{IMP-1} gene cassette, which encodes the IMP-1 metallo-β-lactamase, confers resistance to all β-lactams except monobactams (2, 27, 35). The *aac(6')-Iae* gene cassette, which encodes AAC(6')-Iae, confers resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin (Table 3). The variant *aadA1* gene cassette, which encodes aminoglycoside 3″-adenylyltransferase, confers resistance to streptomycin (18, 22). The *sul1* gene, which encodes dihydropteroate synthetase type I, confers resistance to sulfamethoxazole (47). Thus, the resistance of IMCJ2.S1 to aztreonam, gentamicin, tetracycline, trimethoprim, and silver sulfadiazine appears to be related to another, unidentified resistance factor(s).

In conclusion, we describe here a novel aminoglycoside 6′-*N*-acetyltransferase gene contained on a class 1 integron in a *P. aeruginosa* strain that caused a nosocomial outbreak of urinary tract infections. In113 may spread across Japan, because β-lactams, including carbapenems and aminoglycosides, are frequently used as therapeutic agents against *P. aeruginosa* and methicillin-resistant *Staphylococcus aureus* (20, 23). Surveillance for multidrug-resistant *P. aeruginosa* containing In113 is under way at several medical care facilities in the Sendai area of Japan.

ACKNOWLEDGMENTS

We thank M. Nakano (Jichi Medical School, Japan) for comments on the manuscript, J. Ishikawa and K. Ishino (National Institute of Infectious Diseases, Tokyo, Japan) for AAC(6′) and for advice on an assay of its activities, and S. Iyobe (Kitasato University, Sagamihara, Japan) for *P. aeruginosa* GN17203.

This study was supported by Health Sciences Research grants from the Ministry of Health, Labor, and Welfare of Japan (H15-SHINKO-11 and H16-TOKUBETSU-027).

REFERENCES

1. **Akasaka, T., M. Tanaka, A. Yamaguchi, and K. Sato.** 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. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39:** 1612–1615.
3. **Brown, H. J., H. W. Stokes, and R. M. Hall.** 1996. The integrons In0, In2, and In5 are defective transposon derivatives. J. Bacteriol. **178:**4429–4437.
4. **Centrón, D., and P. H. Roy.** 1998. Characterization of the 6′-*N*-aminoglycoside acetyltransferase gene *aac(6′)-Iq* from the integron of a natural multiresistance plasmid. Antimicrob. Agents Chemother. **42:**1506–1508.
5. **Chow, J. W., V. Kak, I. You, S. J. Kao, J. Petrin, D. B. Clewell, S. A. Lerner, G. H. Miller, and K. J. Shaw.** 2001. Aminoglycoside resistance genes *aph(2″)-Ib* and *aac(6′)-Im* detected together in strains of both *Escherichia coli* and *Enterococcus faecium*. Antimicrob. Agents Chemother. **45:**2691–2694.
6. **Cohen, S. N., A. C. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
7. **Collis, C. M., and R. M. Hall.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob. Agents Chemother. **39:**155–162.
8. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. **174:**1574–1585.
9. **Costa, Y., M. Galimand, R. Leclercq, J. Duval, and P. Courvalin.** 1993. Characterization of the chromosomal *aac(6′)-Ii* gene specific for *Enterococcus faecium*. Antimicrob. Agents Chemother. **37:**1896–1903.
10. **Doi, Y., J. Wachino, K. Yamane, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Spread of novel aminoglycoside resistance gene *aac(6′)-Iad* among *Acinetobacter* clinical isolates in Japan. Antimicrob. Agents Chemother. **48:**2075–2080.
11. **Domenico, P., J. L. Marx, P. E. Schoch, and B. A. Cunha.** 1992. Rapid plasmid DNA isolation from mucoid gram-negative bacteria. J. Clin. Microbiol. **30:**2859–2863.
12. **Dyda, F., D. C. Klein, and A. B. Hickman.** 2000. GCN5-related *N*-acetyltransferases: a structural overview. Annu. Rev. Biophys. Biomol. Struct. **29:**81–103.
13. **Fluit, A. C., and F. J. Schmitz.** 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur. J. Clin. Microbiol. Infect. Dis. **18:**761–770.
14. **Grundmann, H., C. Schneider, D. Hartung, F. D. Daschner, and T. L. Pitt.** 1995. Discriminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. J. Clin. Microbiol. **33:**528–534.
15. **Hall, R., and C. M. Collis.** 1998. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. Drug Resist. Updates **1:**109–119.
16. **Hall, R. M., H. J. Brown, D. E. Brookes, and H. W. Stokes.** 1994. Integrons found in different locations have identical 5′ ends but variable 3′ ends. J. Bacteriol. **176:**6286–6294.
17. **Hall, R. M., and C. M. Collis.** 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol. Microbiol. **15:**593–600.
18. **Hall, R. M., and C. Vockler.** 1987. The region of the IncN plasmid R46 coding for resistance to beta-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn*21*-like transposons. Nucleic Acids Res. **15:**7491–7501.
19. **Hannecart-Pokorni, E., F. Depuydt, L. de Wit, E. van Bossuyt, J. Content, and R. Vanhoof.** 1997. Characterization of the 6′-*N*-aminoglycoside acetyltransferase gene *aac(6′)-Im* [corrected] associated with a *sulI*-type integron. Antimicrob. Agents Chemother. **41:**314–318. (Erratum, **42:**485, 1998.)
20. **Hayashi, I., M. Inoue, and H. Hashimoto.** 1994. Nationwide investigation in Japan on the efficacy of arbekacin in methicillin-resistant *Staphylococcus aureus* infections. Drugs Exp. Clin. Res. **20:**225–232.
21. **Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plesiat.** 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. **47:**1887–1894.
22. **Hollingshead, S., and D. Vapnek.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenylyltransferase. Plasmid **13:**17–30.
23. **Ishihara, S., T. Yamada, S. Yokoi, M. Ito, M. Yasuda, M. Nakano, Y. Kawada, and T. Deguchi.** 2002. Antimicrobial activity of imipenem against isolates from complicated urinary tract infections. Int. J. Antimicrob. Agents **19:**565–569.
24. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
25. **Kato, T., Y. Sato, S. Iyobe, and S. Mitsuhashi.** 1982. Plasmid-mediated gentamicin resistance of *Pseudomonas aeruginosa* and its lack of expression in *Escherichia coli*. Antimicrob. Agents Chemother. **22:**358–363.
26. **Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan.** 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.
27. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J. M. Frere, and G. M. Rossolini.** 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
28. **Lee, K., J. B. Lim, J. H. Yum, D. Yong, Y. Chong, J. M. Kim, and D. M. Livermore.** 2002. bla_{VIM-2} cassette-containing novel integrons in metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolates disseminated in a Korean hospital. Antimicrob. Agents Chemother. **46:**1053–1058.
29. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
30. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
31. **Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**62–66.
32. **Nakaya, R., A. Nakamura, and Y. Murata.** 1960. Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. **3:**654–659.

33. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard. NCCLS document M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
34. **Neuwald, A. F., and D. Landsman.** 1997. GCN5-related histone *N*-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. Trends Biochem. Sci. **22:**154–155.
35. **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.
36. **Partridge, S. R., H. J. Brown, and R. M. Hall.** 2002. Characterization and movement of the class 1 integron known as Tn*2521* and Tn*1405*. Antimicrob. Agents Chemother. **46:**1288–1294.
37. **Partridge, S. R., C. M. Collis, and R. M. Hall.** 2002. Class 1 integron containing a new gene cassette, *aadA10*, associated with Tn*1404* from R151. Antimicrob. Agents Chemother. **46:**2400–2408.
38. **Partridge, S. R., and R. M. Hall.** 2003. In34, a complex In5 family class 1 integron containing *orf513* and *dfrA10*. Antimicrob. Agents Chemother. **47:** 342–349.
39. **Paulsen, I. T., T. G. Littlejohn, P. Radstrom, L. Sundstrom, O. Skold, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
40. **Recchia, G. D., and R. M. Hall.** 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. **5:**389–394.
41. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
43. **Shimizu, K., T. Kumada, W. C. Hsieh, H. Y. Chung, Y. Chong, R. S. Hare, G. H. Miller, F. J. Sabatelli, and J. Howard.** 1985. Comparison of aminoglycoside resistance patterns in Japan, Formosa, and Korea, Chile, and the United States. Antimicrob. Agents Chemother. **28:**282–288.
44. **Shmara, A., N. Weinsetel, K. J. Dery, R. Chavideh, and M. E. Tolmasky.** 2001. Systematic analysis of a conserved region of the aminoglycoside 6′-*N*-acetyltransferase type Ib. Antimicrob. Agents Chemother. **45:**3287–3292.
45. **Stokes, H. W., D. B. O'Gorman, G. D. Recchia, M. Parsekhian, and R. M. Hall.** 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. **26:**731–745.
46. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. 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, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
47. **Swedberg, G.** 1987. Organization of two sulfonamide resistance genes on plasmids of gram-negative bacteria. Antimicrob. Agents Chemother. **31:**306–311.
48. **Tenover, F. C., D. Filpula, K. L. Phillips, and J. J. Plorde.** 1988. Cloning and sequencing of a gene encoding an aminoglycoside 6′-*N*-acetyltransferase from an R factor of *Citrobacter diversus*. J. Bacteriol. **170:**471–473.
49. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
50. **Vakulenko, S. B., and S. Mobashery.** 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. **16:**430–450.
51. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**147–151.
52. **Wright, G. D., and P. Ladak.** 1997. Overexpression and characterization of the chromosomal aminoglycoside 6′-*N*-acetyltransferase from *Enterococcus faecium*. Antimicrob. Agents Chemother. **41:**956–960.
53. **Zhu, C. B., A. Sunada, J. Ishikawa, Y. Ikeda, S. Kondo, and K. Hotta.** 1999. Role of aminoglycoside 6′-acetyltransferase in a novel multiple aminoglycoside resistance of an actinomycete strain #8: inactivation of aminoglycosides with 6′-amino group except arbekacin and neomycin. J. Antibiot. (Tokyo) **52:**889–894.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2005, p. 3948–3951 Vol. 49, No. 9
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.9.3948–3951.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Cloning and Characterization of a Novel Trimethoprim-Resistant Dihydrofolate Reductase from a Nosocomial Isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454)

Jun-ichiro Sekiguchi,[1] Prasit Tharavichitkul,[3] Tohru Miyoshi-Akiyama,[1] Vena Chupia,[3] Tomoko Fujino,[1] Minako Araake,[1] Atsushi Irie,[1] Koji Morita,[4] Tadatoshi Kuratsuji,[2,5] and Teruo Kirikae[1]*

Department of Infectious Diseases[1] and Research Institute,[2] International Medical Center of Japan, Shinjuku, Tokyo 162-8655, Japan; Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand[3]; Department of Microbiology, Kyorin University School of Health Sciences, Hachioji, Tokyo 192-8508, Japan[4]; and National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan[5]

Received 16 March 2005/Returned for modification 24 April 2005/Accepted 14 June 2005

A novel gene, *dfrG*, encoding a trimethoprim (TMP)-resistant dihydrofolate reductase (DHFR, designated S3DHFR) was cloned from a clinical isolate of methicillin-resistant *Staphylococcus aureus*. *Escherichia coli* expressing *dfrG* was highly resistant to TMP. Recombinant S3DHFR exhibited DHFR activity that was not inhibited by TMP.

Trimethoprim (TMP) is a potent inhibitor of bacterial dihydrofolate reductase (DHFR) and is effective in vitro against methicillin-resistant *Staphylococcus aureus* (MRSA). In combination with sulfamethoxazole, TMP has been used successfully to treat patients infected with MRSA and is effective at eradicating carriage (10, 16). Resistance of *S. aureus* to TMP was first reported in the 1980s (12) and was found to be due to plasmid-mediated production of an additional DHFR that was less sensitive to TMP than intrinsic DFHR (*S. aureus* DHFR [SaDHFR]) encoded by the *dfrB* gene on the chromosome (1, 12). Plasmid-mediated production of an additional TMP-resistant DHFR is one of the most common mechanisms of resistance to TMP in bacterial organisms. At least 14 different types of TMP-resistant DHFRs in gram-negative bacteria have been reported (10); however, only a limited number of TMP-resistant DHFRs in gram-positive bacteria have been reported (10).

A total of 43 clinical isolates of MRSA from Chiang Mai, Thailand, and 244 clinical isolates of MRSA from Tokyo, Japan, were analyzed in this study. All isolates were positive for *dfrB* by PCR and also positive for *femB* encoding coagulase and for *mecA* associated with methicillin resistance. All isolates from Chiang Mai, Thailand, were resistant to TMP, whereas all those from Tokyo, Japan, except one, *S. aureus* IMCJ934, were sensitive to TMP (Table 1). Crude extracts prepared from a TMP-resistant isolate from Chiang Mai, *S. aureus* CM.S2 (IMCJ1454), showed DHFR activity, and K_m values of the extract for DHF and NADPH were similar to those of crude extracts from TMP-sensitive strain ATCC 25923 (Table 2); however, the 50% inhibitory concentration (IC_{50}) of TMP for the crude extract of strain CM.S2 was more than 15,000-fold greater than that of ATCC 25923.

HindIII-digested fragments of the *S. aureus* CM.S2 genome were cloned, transformed into *Escherichia coli* DH5α cells, and selected on agar medium containing TMP (8 μg/ml). The resultant plasmid, named pSA1, had a 3.5-kb insert containing a complete open reading frame (ORF) surrounded by truncated ORFs (data not shown). The complete ORF consisted of 498 bp encoding a putative protein of 165 amino acids with similarities to TMP-resistant DHFR from *Staphylococcus haemolyticus* (79% identity) (7), *Bacillus anthracis* (67% identity) (2), and *Bacillus cereus* (65% identity) (15) (Fig. 1). The deduced

TABLE 1. MICs of trimethoprim in *S. aureus* and *E. coli* strains

Strain	MIC of TMP (μg/ml)	Characteristic(s) or genotype
S. aureus CM.S2 (IMCJ1454)	>512	Clinical isolate from Chiang Mai, Thailand, in 2003
S. aureus IMCJ934	>512	Clinical isolate from Tokyo, Japan, in 2001
S. aureus ATCC 29213	4	Quality control strain for antimicrobial susceptibility testing
E. coli DH5α(pSA1)	>512	Transformant harboring a 3.5-kb BamHI fragment with *dfrG* ligated to pHSG398
E. coli DH5α(pHSG398)	<2	Transformant harboring pHSG398
E. coli DH5α(pT7dfrG)	>512	Transformant harboring PCR-amplified *dfrG* ligated to pCRT7/NT
E. coli DH5α(pT7dfrB)	128	Transformant harboring PCR-amplified intrinsic *dfrB* ligated to pCRT7/NT
E. coli DH5α(pCRT7/NT)	<2	Transformant harboring pCRT7/NT
E. coli DH5α	<2	*supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1*

* Corresponding author. Mailing address: Department of Infectious Diseases, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. Phone: (81) 3 3202 7181, ext. 2838. Fax: (81) 3 3202 7364. E-mail: tkirikae@ri.imcj.go.jp.

TABLE 2. Enzyme kinetic and inhibitory properties of staphylococcal DHFRs

DHFR	Origin	K_m (μM)		IC_{50} of TMP (μM)
		DHF	NADPH	
Crude enzyme	*S. aureus* CM.S2 (IMCJ1454)	5.83 ± 2.09	15.17 ± 1.73	214
	S. aureus ATCC 25923	3.16 ± 1.99	14.78 ± 2.73	0.013
TMP-resistant DHFRs				
S3DHFR	*S. aureus* CM.S2 (IMCJ1454)	2.68 ± 1.09	2.38 ± 1.97	254
S2DHFR[a]	*S. haemolyticus* MUR313	5.1	1.7	127
S1DHFR[a]	*S. aureus*	6.6	12.4	9.8
TMP-sensitive DHFRs				
$SaDHFR_{CM.S2}$	*S. aureus* CM.S2 (IMCJ1454)	3.01 ± 1.40	2.97 ± 0.57	0.014
SaDHFR[a]	*S. aureus* ATCC 25923			0.012

[a] Data from references 6, 7, and 8.

protein is somewhat less similar to the intrinsic TMP-sensitive DHFRs from *S. aureus* (SaDHFR) (8), *S. epidermidis* (SeDHFR) (6), and *E. coli* K-12 (17), with 41%, 40%, and 40% similarity, respectively (Fig. 1). This complete ORF was named *dfrG*, and the deduced protein was designated S3DHFR. Amino acid sequence alignment of DHFRs suggests that residues involved in the binding of TMP and NADPH in other DHFRs are conserved in S3DHFR (Fig. 1). An ORF downstream of *dfrG*, designated *orfU1*, was located in the opposite direction of *dfrG* and consists of 1,950 bp encoding 650 amino acids, although the deduced amino acid sequence did not show any significant homology to sequences of other previously reported proteins. An ORF upstream of *dfrG* consisted of 582 nucleotides and was identical to the 3′-flanking region of the *SAV0404* gene encoding a hypothetical protein (11). *dfrG* and *orfU1* were flanked by a 28-bp inverted repeat and a 7-bp direct repeat,

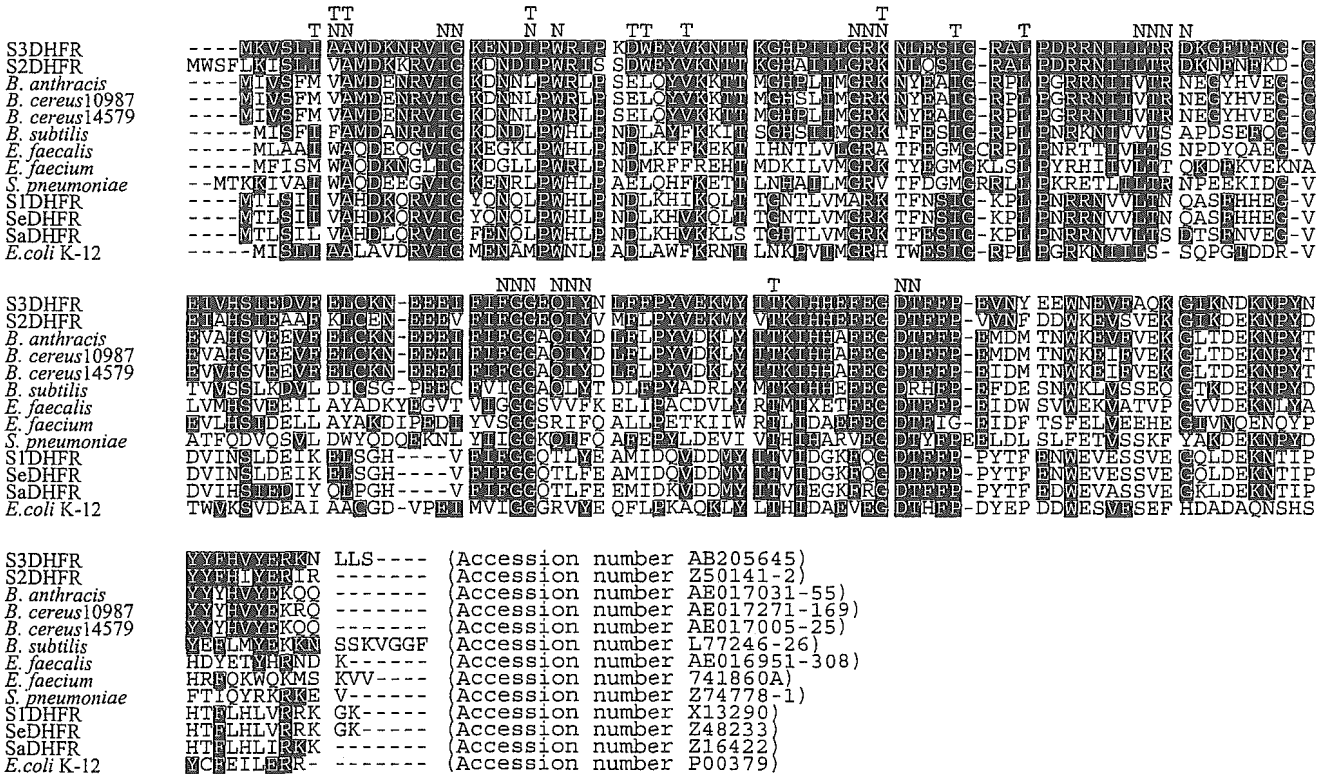

FIG. 1. Multiple-sequence alignment of the amino acid sequence of S3DHFR from *S. aureus* CM.S2 (IMCJ1454) isolate with those of DHFRs from other bacteria. The amino acid sequence of S3DHFR was compared with that of type S1 from *S. aureus*, S2 from *S. haemolyticus* MUR313, and the chromosomal DHFRs from *B. anthracis* Ames, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *Bacillus subtilis* Marburg, *E. faecalis* V583, a methotrexate-resistant mutant of *E. faecium* strain A, *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus epidermidis* ATCC 14900 (SeDHFR), *S. aureus* ATCC 25923 (SaDHFR), and *E. coli* K-12. Sequence comparison was performed by aligning the proteins with the ClustalW program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Amino acid positions involved in the binding of trimethoprim (T) and NADPH cofactor (N) are according to studies of the *E. coli* K-12 enzyme (3, 9, 13, 14). Identical residues are indicated by white letters on black background. Gaps introduced to maximize alignment are indicated by dashes.

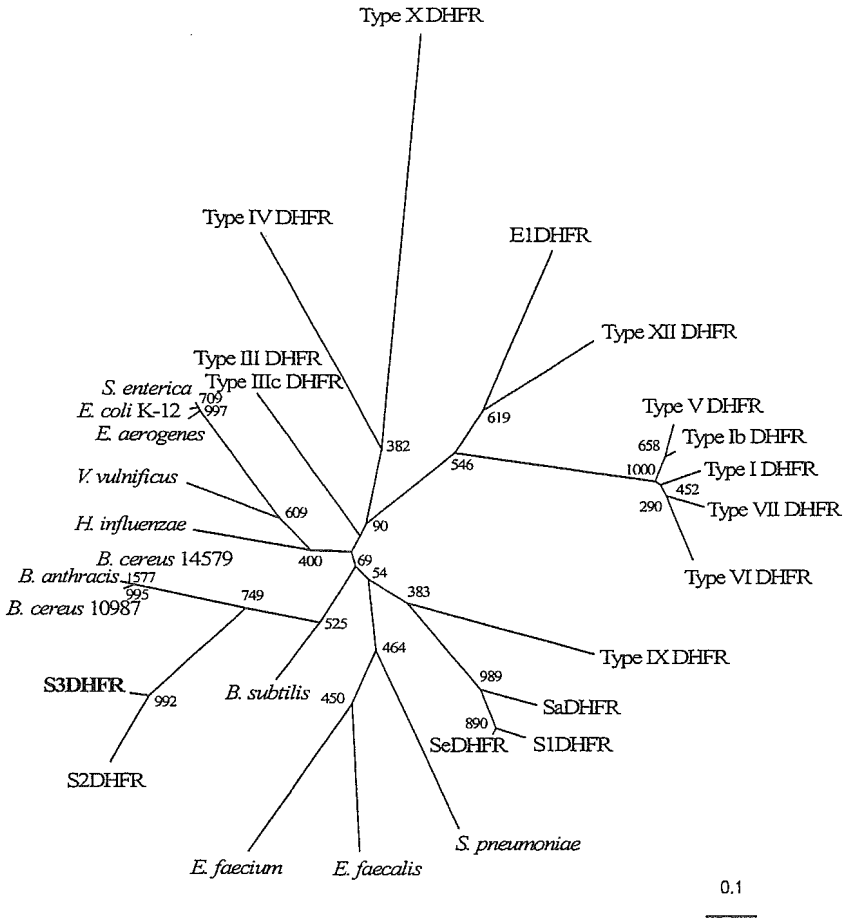

FIG. 2. Dendrogram of S3DHFR and DHFR from a variety of organisms. The dendrogram was created by the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the DHFR proteins (accession number and species given in parentheses) of types I (X00926, from *E. coli*), Ib (I40985, from *E. coli*), IV (A60935, from *E. coli*), V (X12868, from enterobacterial plasmid pLMO150), VI (Z86002, from *Proteus mirabilis*), VII (X58425, from *E. coli*), VIII (U10186, from *E. coli*), IX (A49788, from *E. coli*), X (AY123253, from *Klebsiella pneumoniae*), XII (I41043, from *E. coli*), E1 (AF028812, from *E. faecalis*), S1 (X13290, from *S. aureus*), and S2 (Z50141, from *S. haemolyticus* MUR313) and the chromosomal DHFRs of *B. anthracis* Ames (AE017031), *B. cereus* ATCC 14579 (AE017005), *B. cereus* ATCC 10987 (AE017271), *B. subtilis* Marburg (L77246), *Enterobacter aerogenes* (M26022), *E. coli* K-12 (P00379), *E. faecalis* V583 (AE016951), *E. faecium* mutant strain A (741860A), *Haemophilus influenzae* R1047 (X84205), *Salmonella enterica* serovar Paratyphi ATCC 9150 (CP000026), *S. aureus* ATCC 25923 (SaDHFR; Z16422), *S. epidermidis* ATCC 14900 (SeDHFR; Z48233), *Streptococcus pneumoniae* ATCC 49619 (Z74778), and *Vibrio vulnificus* YJ016 (BA000037).

indicating that the region is in an insertion sequence (IS). The DNA sequence, ranging from 275 bp upstream of *dfrG* to the 363 bp of the 5′-terminal region of *dfrG*, was identical to that previously reported for plasmid pMG1 in *Enterococcus faecium* (18). The *dfrG* gene may have been acquired from *E. faecium* via IS-mediated recombination. The ancestral origin of S3DHFR, however, remains unknown; S3DHFR showed little similarity to and considerable phylogenetic distance from intrinsic DHFR of *E. faecium* (Fig. 2).

The MICs of TMP in *E. coli* transformants harboring pSA1 or pT7dfrG carrying *dfrG* were significantly increased than those in control strains (Table 1), indicating that *dfrG* is responsible for TMP resistance. An *E. coli* transformant harboring pT7dfrB carrying *dfrB* also showed increased MIC, but it was not as high as those of *E. coli* strains expressing *dfrG*. *dfrB* is believed to encode a TMP-sensitive DHFR of *S. aureus* because it was found in all *S. aureus* strains, regardless of TMP susceptibility. Similar results were reported for *dfrE* encoding *Enterococcus faecalis* DHFR (4). The increased MIC for TMP in *E. coli* carrying *dfrB* may be explained by the multicopy effects of high expression of the housekeeping protein DHFR.

For functional analysis of S3DHFR and DHFR from *S. aureus* CM.S2 ($SaDHFR_{CM.S2}$), overexpression and purification of these recombinant DHFRs were achieved. Overexpression of S3DHFR or $SaDHFR_{CM.S2}$ was accomplished by integration of the respective coding regions downstream of the His-tagged coding region of the pCR/T7NT expression vector and transformation into the *E. coli* strain BL21-AI. Recombinant protein in soluble extracts was purified by affinity chromatography to determine enzymatic activities. The K_m values of recombinant S3DHFR for DHF and NADPH were 2.68 ± 1.09 μM and 2.38 ± 1.97 μM, respectively (Table 2). The K_m values of DHF and NADPH for S3DHFR did not differ from those of $SaDHFR_{CM.S2}$, but the IC_{50} values of TMP for these DHFRs differed significantly. The IC_{50} of TMP for S3DHFR was more than 8,000-fold greater than IC_{50} values for TMP-

sensitive SaDHFR and $SaDHFR_{CM.S2}$, indicating that S3DHFR and $SaDHFR_{CM.S2}$ are indeed DHFRs but that only S3DHFR plays a critical role in TMP resistance. The K_m values of crude extracts for NADPH were sixfold greater than those of recombinant S3DHFR (Table 2). Crude extracts may contain other factor(s) that bind to NADPH.

Detection of *dfrG* was performed by PCR on isolates from Chiang Mai, Thailand, and Tokyo, Japan. All Chiang Mai isolates were resistant to TMP and contained *dfrG*, whereas all Tokyo isolates but one were sensitive to TMP and did not contain *dfrG* (data not shown). The single Tokyo isolate IMCJ934 was resistant to TMP and contained *dfrG* (Table 1).

Pulsed-field gel electrophoresis (PFGE) analysis revealed 13 patterns of SmaI digestion in the 43 MRSA isolates from Chiang Mai, Thailand (data not shown). Cluster analysis showed that 12 of the 13 PFGE patterns formed a cluster (>75% similarity). The PFGE pattern of *S. aureus* CM.S2 genomic DNA was identical to that of 18 MRSA isolates. These results suggest that clonal expansion of MRSA carrying *dfrG* occurred at the hospital in Chiang Mai. The TMP-resistant isolate from Tokyo, Japan, IMCJ934, showed the same PFGE pattern as that of one of the Chiang Mai isolates, *S. aureus* CM.S2 (data not shown).

dfrG was detected by Southern blotting on fragments of SmaI-digested genomic DNA, but it was not detected on plasmids (data not shown). Conjugal transfer of TMP resistance from *S. aureus* CM.S2 to recipient strains *S. aureus* IMCJ565RFP^r or IMCJ644RFP^r was unsuccessful, suggesting that *dfrG* is located on the chromosome and not on a plasmid of these clinical isolates. It remains to be determined whether *dfrG* can be transferred by phages or mobile elements.

A single amino acid substitution (Phe to Tyr) at codon 98 of SaDHFR was reported to be associated with TMP resistance in *S. aureus* (5). Therefore, approximately 390 bp of internal DNA sequence of *dfrB* encoding SaDHFR was determined. When *S. aureus* ATCC 29213 was used as a control (5), all isolates from Chiang Mai, Thailand, exhibited three silent mutations: CAT to CAC in codon 77 and TTT to TTC in codons 91 and 118. All isolates from Tokyo, Japan, contained four silent mutations: AAA to AAG in codon 30, CAT to CAC in codon 77, and TTT to TTC in codons 91 and 118. These results indicate that these mutational changes are not associated with TMP resistance in the isolates from Chiang Mai or Tokyo. Other possible mechanisms of TMP resistance, such as overexpression of intrinsic DHFR, efflux, or impermeability, may be involved.

The CM.S2 strain was the dominant clone from Chiang Mai, Thailand. MRSA surveillance is being carried out in the hospital from which these isolates were obtained. *S. aureus* CM.S2 is resistant to clindamycin, erythromycin, gentamicin, and tetracycline and is less sensitive to arbekacin. Fosfomycin, linezolid, and vancomycin are effective in vitro; quinupristin-dalfopristin and daptomycin were not available for testing. Results of this surveillance will be reported in the future.

Our data strongly suggest that the TMP resistance-associated gene *dfrG* is prevalent in Thailand, and an isolate harboring this gene was found in Japan. This gene may spread worldwide, and measures against this, such as gene monitoring and adequate use of TMP, should be established.

This study was supported by Health Sciences research grants from the Ministry of Health, Labor and Welfare (H15-SHINKO-11).

REFERENCES

1. **Archer, G. L., J. P. Coughter, and J. L. Johnston.** 1986. Plasmid-encoded trimethoprim resistance in staphylococci. Antimicrob. Agents Chemother. **29:**733–740.
2. **Barrow, E. W., P. C. Bourne, and W. W. Barrow.** 2004. Functional cloning of *Bacillus anthracis* dihydrofolate reductase and confirmation of natural resistance to trimethoprim. Antimicrob. Agents Chemother. **48:**4643–4649.
3. **Bolin, J. T., D. J. Filman, D. A. Matthews, R. C. Hamlin, and J. Kraut.** 1982. Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 A resolution. I. General features and binding of methotrexate. J. Biol. Chem. **257:**13650–13662.
4. **Coque, T. M., K. V. Singh, G. M. Weinstock, and B. E. Murray.** 1999. Chazracterization of dihydrofolate reductase genes from trimethoprim-susceptible and trimethoprim-resistant strains of *Enterococcus faecalis*. Antimicrob. Agents Chemother. **43:**141–147.
5. **Dale, G. E., C. Broger, A. D'Arcy, P. G. Hartman, R. DeHoogt, S. Jolidon, I. Kompis, A. M. Labhardt, H. Langen, H. Locher, M. G. Page, D. Stuber, R. L. Then, B. Wipf, and C. Oefner.** 1997. A single amino acid substitution in *Staphylococcus aureus* dihydrofolate reductase determines trimethoprim resistance. J. Mol. Biol. **266:**23–30.
6. **Dale, G. E., C. Broger, P. G. Hartman, H. Langen, M. G. Page, R. L. Then, and D. Stuber.** 1995. Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of *Staphylococcus epidermidis* ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from *Staphylococcus aureus*? J. Bacteriol. **177:**2965–2970.
7. **Dale, G. E., H. Langen, M. G. Page, R. L. Then, and D. Stuber.** 1995. Cloning and characterization of a novel, plasmid-encoded trimethoprim-resistant dihydrofolate reductase from *Staphylococcus haemolyticus* MUR313. Antimicrob. Agents Chemother. **39:**1920–1924.
8. **Dale, G. E., R. L. Then, and D. Stuber.** 1993. Characterization of the gene for chromosomal trimethoprim-sensitive dihydrofolate reductase of *Staphylococcus aureus* ATCC 25923. Antimicrob. Agents Chemother. **37:**1400–1405.
9. **Filman, D. J., J. T. Bolin, D. A. Matthews, and J. Kraut.** 1982. Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 A resolution. II. Environment of bound NADPH and implications for catalysis. J. Biol. Chem. **257:**13663–13672.
10. **Huovinen, P., L. Sundstrom, G. Swedberg, and O. Skold.** 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother. **39:**279–289.
11. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
12. **Lyon, B. R., J. W. May, and R. A. Skurray.** 1983. Analysis of plasmids in nosocomial strains of multiple-antibiotic-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **23:**817–826.
13. **Matthews, D. A., J. T. Bolin, J. M. Burridge, D. J. Filman, K. W. Volz, B. T. Kaufman, C. R. Beddell, J. N. Champness, D. K. Stammers, and J. Kraut.** 1985. Refined crystal structures of *Escherichia coli* and chicken liver dihydrofolate reductase containing bound trimethoprim. J. Biol. Chem. **260:**381–391.
14. **Matthews, D. A., J. T. Bolin, J. M. Burridge, D. J. Filman, K. W. Volz, and J. Kraut.** 1985. Dihydrofolate reductase. The stereochemistry of inhibitor selectivity. J. Biol. Chem. **260:**392–399.
15. **Rasko, D. A., J. Ravel, O. A. Okstad, E. Helgason, R. Z. Cer, L. Jiang, K. A. Shores, D. E. Fouts, N. J. Tourasse, S. V. Angiuoli, J. Kolonay, W. C. Nelson, A. B. Kolsto, C. M. Fraser, and T. D. Read.** 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Res. **32:**977–988.
16. **Roccaforte, J. S., M. J. Bittner, C. A. Stumpf, and L. C. Preheim.** 1988. Attempts to eradicate methicillin-resistant *Staphylococcus aureus* colonization with the use of trimethoprim-sulfamethoxazole, rifampin, and bacitracin. Am. J. Infect. Control. **16:**141–146.
17. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8:**2255–2274.
18. **Tanimoto, K., and Y. Ike.** 2002. Analysis of the conjugal transfer system of the pheromone-independent highly transferable *Enterococcus* plasmid pMG1: identification of a *tra* gene (*traA*) up-regulated during conjugation. J. Bacteriol. **184:**5800–5804.

CHEST®

THE CARDIOPULMONARY
AND CRITICAL CARE JOURNAL

FOR PULMONOLOGISTS, CARDIOLOGISTS, CARDIOTHORACIC SURGEONS,
CRITICAL CARE PHYSICIANS, AND RELATED SPECIALISTS

Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation

Wei Xiao, Yao-Pi Hsu, Akitoshi Ishizaka, Teruo Kirikae and Richard B. Moss

Chest 2005;128;2316-2326
DOI: 10.1378/chest.128.4.2316

This information is current as of March 27, 2006

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://www.chestjournal.org/cgi/content/full/128/4/2316

CHEST is the official journal of the American College of Chest Physicians. It has been published monthly since 1935. Copyright 2005 by the American College of Chest Physicians, 3300 Dundee Road, Northbrook IL 60062. All rights reserved. No part of this article or PDF may be reproduced or distributed without the prior written permission of the copyright holder.
ISSN: 0012-3692.

AMERICAN COLLEGE OF
CHEST
PHYSICIANS

Downloaded from www.chestjournal.org at Kokuritsu-Kokusai-Iryo-Center on March 27, 2006

Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation*

Wei Xiao, MD; Yao-Pi Hsu, MS; Akitoshi Ishizaka, MD; Teruo Kirikae, MD, PhD; and Richard B. Moss, MD

Background: **Interest in airways inflammatory disease has increasingly focused on innate immunity. We investigated several components of innate immunity in induced sputum of patients with cystic fibrosis (CF), COPD, and asthma, and healthy control subjects.**

Methods: **Twenty eight patients with mild CF lung disease (age ≥ 12 years; FEV_1, 74 ± 3% predicted [mean ± SE]), 74 adults with COPD (FEV_1, 55 ± 2% of predicted), 34 adults with persistent asthma (FEV_1, 66 ± 2% of predicted), and 44 adult control subjects (FEV_1, 85 ± 1% of predicted) were studied while in stable clinical condition. Levels of sputum interleukin (IL)-8, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, human cationic antimicrobial protein 18 (CAP18), urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor (PAI)-1 were determined. Cell sources were investigated by flow cytometry and immunohistochemistry. Spirometry was performed prior to sputum induction.**

Results: **CF patient sputum showed greatest increase in IL-8 compared to that of patients with COPD and asthma (which were also greater than control subjects), and elevated levels of TNF-α and IL-10 compared to other groups. There were no differences in IFN-γ. CAP18 levels were elevated in CF and COPD patients compared to control subjects, while asthma patients had reduced CAP18 levels. uPA levels were similar but uPAR was elevated in CF and COPD patients more so than in asthma patients, while PAI-1 levels were elevated in all three disease groups. CAP18 localized to neutrophil secondary granules; neutrophils were also sources of IL-8 and PAI-1. CAP18 and PAI-1 negatively correlated with pulmonary function.**

Conclusion: **Induced-sputum innate immune factor levels discriminate inflammatory changes in CF, COPD, and asthma, suggesting potential roles in pathophysiology and as well as providing disease-specific biomarker patterns.** ***(CHEST 2005; 128:2316–2326)***

Key words: cathelicidin; cystic fibrosis; cytokine; innate immunity; urokinase plasminogen activator system

Abbreviations: BSA = bovine serum antigen; CAP18 = human cationic antimicrobial protein 18; CF = cystic fibrosis; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; PAI = plasminogen activator inhibitor; PBS = phosphate-buffered saline solution; PE = phycoerythrin; TNF = tumor necrosis factor; uPA = urokinase-type plasminogen activator; uPAR = uPA receptor

The pathogenesis of cystic fibrosis (CF) lung disease is characterized by compromised local innate immunity, which permits microbial colonization and chronic infection.[1] Current thinking emphasizes the primary role of volume depletion of airway surface liquid and resulting compromise of mucociliary clearance.[2] Additional innate defense mechanisms may also be involved, as a primary proinflam-

*From the Department of Medicine (Dr. Xiao), Shandong University, Shandong, Jinan, Peoples Republic of China; Department of Pediatrics (Mrs. Hsu and Dr. Moss), Stanford University, Stanford CA; Department of Medicine (Dr. Ishizaka), Keio University, Tokyo, Japan; and Department of Infectious Diseases and Tropical Medicine (Dr. Kirikae), International Medical Center of Japan, Tokyo, Japan.

This study was funded by the Ross Mosier and Berger Raynolds Funds.

Drs. Moss, Ishizaka, and Kirikae have assigned entire right, title, and interest in the CAP18 immunoassay described here to Seikagaku Corporation, Tokyo, Japan.

Manuscript received January 27, 2005; revision accepted April 29, 2005.

Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (www.chestjournal.org/misc/reprints.shtml).

Correspondence to: Richard Moss, MD, Pediatric Pulmonary Medicine, Stanford University Medical Center, 701A Welch Rd #3328, Palo Alto, CA 94304-5786; e-mail: rmoss@stanford.edu

Downloaded from www.chestjournal.org at Kokuritsu-Kokusai-Iryo-Center on March 27, 2006

matory bias of the CF epithelia has been posited,[3-4] and activity of endogenous antimicrobial peptides produced by airway epithelia and glands may be altered in CF.[5-6] While some epithelial antimicrobials have received much attention in CF, in particular the β-defensins, others have not.[7-8] In particular, the role of the only human cathelicidin, the 18-kd, 140 amino acid cationic antimicrobial protein (human cationic antimicrobial protein 18 [CAP18]), has not been investigated in sputum from patients with CF. It is produced by respiratory epithelia as well as stored in secondary (specific) granules of polymorphonuclear leukocytes, and in a model system restores deficient antimicrobial activity in the CF airway milieu.[9-10] Proteolytic cleavage of CAP18 by proteinase 3 yields a potent antimicrobial peptide (carboxy terminal 37 amino acid fragment of CAP18).[11] Via its action on the formyl peptide receptor-like 1 expressed on several cell types, carboxy terminal 37 amino acid fragment of CAP18 is also an important regulator of macrophage function; has potent chemotactic activity for neutrophils, monocytes, and T cells; and possesses angiogenesis activity.[12-14] Abnormalities in CAP18 could therefore profoundly affect the pathophysiology of CF by its ability to link innate to adaptive immunity and its neovascularizing effect.

Another innate defense pathway recently found active in the airway is the plasminogen activator system constituted by its local serine protease activator urokinase-type plasminogen activator (uPA), the uPA-specific cell surface receptor (uPA receptor [uPAR]) [CD87], and an arginine-specific serine protease inhibitor (serpin), plasminogen activator inhibitor (PAI)-1.[15-16] uPA binding to uPAR results in enhanced activation of cell-bound plasminogen with subsequent effects on cell adhesion, chemotactic migration, and tissue remodeling.[16-20] The uPA system is involved in a number of pathologic states, including inflammation after tissue injury as key participants in the enzymatic modification of the extracellular matrix, resulting in cell recruitment, migration, adhesion, and mitogenesis.[15,19] In addition to proteolytic activity, plasmin activates metalloproteinases that degrade extracellular matrix.[20] To maintain normal lung function and integrity in the host response, tight control of the proteolytic enzymes and their inhibitors is needed to maintain proper function of neutrophils, macrophages, and mesenchymal cells. The uPA system is one of the components that act on neutrophils and macrophages to facilitate the interaction between cells and matrix.[21-22] A critical innate defense role for the uPA/uPAR/PAI-1 system has recently been described in murine models of acute pulmonary infection as well as in the pathogenesis of interstitial lung diseases.[23-26] The role of this system in human chronic airway inflammatory lung diseases is just emerging.[26-27]

CF, COPD, and asthma are chronic airway diseases with different causes that share some features in pathologic changes and clinical syndrome. In these diseases, there is chronic mucosal and airway inflammation with distinct pathophysiologic features in each but a common increase in the infiltration of neutrophils and a variety of inflammatory mediators including interleukin (IL)-8. The pathologic processes in these diseases all seem to involve progressive inflammatory responses with elements of tissue remodeling, airway obstruction, and reduction in expiratory flow rates. In the present study, we determined and compared the levels and sources of uPA/uPAR/PAI-1, CAP18, and several cytokines (IL-8, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and IL-10) in induced sputum of CF, COPD, and asthma patients, and healthy control subjects to see if differing local inflammatory patterns can be discerned noninvasively and related to airflow obstruction as measured by expiratory flow rates.

Materials and Methods

Subjects

We recruited 28 patients with CF (age, 12 to 50 years) followed up at the CF Center clinic at Stanford University Medical Center. CF diagnoses in all patients were made by positive (> 60 mEq/L) pilocarpine iontophoresis sweat test results, with homozygous or compound heterozygous for ΔF508 CF transmembrane conductance regulator mutations. All patients had chronic infection with *Pseudomonas aeruginosa* by serial sputum culture and were in stable clinical condition (no pulmonary exacerbation within previous month). CF patients were also excluded for FEV_1 values < 40% predicted, oxyhemoglobin saturation < 92% on room air, pneumothorax, hemoptysis, or history of *Burkholderia cepacia* in sputum. None were receiving regular inhaled or systemic corticosteroids. Seventy-four patients (age 38 to 79 years) with previously diagnosed COPD were recruited from the Respiratory Clinic of the Hospital of Shandong Medical University, Peoples Republic of China. All were in stable condition and not receiving antibiotics for at least 2 weeks prior to testing. Thirty-four patients with asthma (age, 14 to 75 years) followed up in Shandong were also studied. Finally 44 healthy nonsmoking subjects without reported respiratory symptoms (age, 20 to 60 years; 20 patients at Stanford and 24 patients at Shandong) were also studied. All participants gave written informed consent with protocols approved by the Institutional Review Boards at Stanford and Shandong.

Pulmonary Function

Pulmonary function tests were performed according to American Thoracic Society guidelines for performance and acceptance prior to sputum induction.

Downloaded from www.chestjournal.org at Kokuritsu-Kokusai-Iryo-Center on March 27, 2006

Sputum Induction and Processing

Sputum was collected from each patient as previously described using 3% hypertonic saline solution (at Stanford) or 3.5% hypertonic saline solution (at Shandong) via an ultrasonic nebulizer with 2-min collections of sputum, which were pooled for analysis.[26–29] All subjects underwent sputum induction regardless of history of ability to expectorate. Subjects were encouraged to cough, and sputum was collected into polypropylene cups. The induced-sputum samples were weighed, and an equal volume of Sputolysin (Calbiochem-Novabiochem; San Diego CA) diluted 10% in normal saline solution was added. Samples were vortexed 3 seconds and incubated for 5 min at 37°C in a water bath with vigorous shaking (160 rotations per minute). Samples were further mixed by aspirating up and down 20 times in a transfer pipette. Five-minute incubations were then repeated two more times. Finally, the samples were centrifuged at 2,000 revolutions per minute (800g) for 5 min at 4°C, and the sol phase was used for analysis.

Soluble Mediators of Innate Immunity

Human IL-8, IL-10, IFN-γ, TNF-α, CAP18, uPA, uPAR, and PAI-1 levels in the supernatant sol phase of sputum were determined by enzyme-linked immunosorbent assay (ELISA) using a standardized format. Wells of microtiter plates (polypropylene 96-well culture clusters, Catalog No. 3598; Costar; Pleasanton CA) were coated with 50 μL per well capture antibody (see below for specific reagents) diluted in phosphate-buffered saline solution (PBS) [P-4417; Sigma Chemical; St. Louis, MO], incubated overnight at 4°C, and washed three times with wash buffer (PBS 0.01%, Thimersol; Sigma Chemicals; 0.05% Tween 20 [polyoxyethylene sorbitan mono-oleate]; Sigma Chemical). Blocking solution (1% bovine serum albumin [BSA], A-2153; Sigma Chemical; 5% sucrose PBS) 200 μL per well was added, incubated at room temperature for 1 h, and the wells were washed three times with washing buffer. Samples and standards diluted in diluting solution (0.1% BSA-0.05% Tween 20-Tris-buffered saline solution) were then added (50 μL per well), incubated overnight at 4°C, and washed three times with washing buffer. Biotinylated detection antibody (see below for specific reagents) in dilution buffer was then added (50 μL per well) and incubated 2 h at room temperate with gentle mixing. Plates were then washed four times with washing buffer. Avidin-peroxidase-conjugated secondary antibody (see below for specific reagent) diluted in dilution buffer was then added (50 μL per well), and incubated 1 h at room temperature with gentle mixing. Plates were then washed four times with washing buffer. Developing solution (75 μL per well *o*-phenylenediamine [P-6912; Sigma Chemical] in citrate-phosphate buffer pH 6.0 with 4 μL 30% H_2O_2 [H-1009; Sigma Chemical]) per 10-mL buffer was added, the reaction stopped with 25 μL per well 2 N H_2SO_4, and the well color was read at an optical density of 492 with an automated microplate reader (Molecular Devices; Mountain View, CA).

Specific Reagents for the ELISA

IL-10: For IL-10, the capture antibody was from Pharmingen (Catalog No. 18551D; BD Pharmingen; San Diego, CA), the primary antibody was purified rat anti-human IL-10 diluted to 4 μg/mL, the detection antibody was biotinylated rat anti-human IL-10 (Catalog No. 18562D; BD Pharmingen) diluted to 4 μg/mL, the detector was horseradish peroxidase-streptavidin (Catalog No. 43–4323; Zymed Laboratories; San Francisco, CA) diluted 1:1000, and the standard was recombinant human IL-10 (Catalog No. 19701N; BD Pharmingen) diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IFN-γ: For IFN-γ, the capture antibody was purified mouse anti-human INF-γ (N1B4, Catalog No. 18891D; BD Pharmingen) diluted to 2 μg/mL, the detection antibody was biotinylated mouse anti-human IFN-γ (4SB3, Catalog No. 18902D; BD Pharmingen) diluted to 2 μg/mL, and the standard was recombinant human IFN-γ (Catalog No. 19751N; BD Pharmingen), diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IL-8: For IL-8, the capture antibody was purified monoclonal antibody to human IL-8 (Catalog No. MAB208; R&D Systems; Minneapolis, MN) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human IL-8 (Catalog No. BAF208; R&D Systems) diluted to 200 ng/mL, and the standard was recombinant human IL-8 (Catalog No. 208-IL; R&D Systems) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, and 78 pg/mL.

TNF-α: For TNF-α, the capture antibody was mouse anti-human TNF-α (Part 840119; R&D Systems) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human TNF-α (Part 840120; R&D Systems) diluted to 300 ng/mL, the detector was horseradish peroxidase-streptavidin (Part 89080; R&D Systems) diluted to 1:200, and the standard was recombinant human TNF-α (Part 840121; R&D Systems) diluted to 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 16 pg/mL.

uPA, PAI-1, and uPAR: For uPA, PAI-1, and uPAR, ELISA kits (Imubind; American Diagnostics; Greenwich, CT) were used according to the instructions of the manufacturer (Catalog Nos. 894, 821, and 893, respectively).

CAP18: For CAP18, the capture antibody was rabbit polyclonal antibody to human lipopolysaccharide-binding domain of CAP18 diluted to 1:200; the detection antibody was a mouse IgG1 monoclonal antibody to CAP18 diluted to 1:1000; the detector was horseradish peroxidase-conjugated goat anti-mouse IgG (Catalog No. 115-367-5296; Jackson Immuno Research Lab Inc.; West Grove, PA) diluted 1:2500; and the standard was a synthetic 27 amino acid peptide fragment of CAP18 (amino acids 109–135) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, 78, 39, and 20 ng/mL. The capture antibody, detection antibody and standard were obtained from Dr. Yoshikazu Naiki and Teruo Kirikae, Japan. In a pilot study, the same CAP18 ELISA was used to measure CAP18 levels in serum, BAL fluid, and expectorated sputum of patients with CF (see "Results").

Immunhistochemistry of Induced-Sputum Cells

Adherence of Sputum Cells to Glass Slides: After removal of the supernatant of centrifuged sputum samples, cell pellets were each suspended in 20 mL of PBS (P-4417; Sigma Chemical) and centrifuged at 1,200 revolutions per min for 10 min. After removal of the supernatant, the cell pellet was resuspended in 1% BSA (A-2153; Sigma Chemical)-PBS. Dead cells were excluded by Trypan Blue (T-9520; Sigma Chemical) cell count. Cells were diluted to approximately 2.5×10^5 in 1% BSA-PBS. Following cytocentrifuge (700 revolutions per minute for 5 min) 100 μL of cell suspension per slide were air dried and freezer stored at −20°C.

Staining: Frozen slides were thawed at room temperature. Cells were fixed by incubating slides in 4% formaldehyde (Catalog No. 16220; Electron Microscopy Sciences; Fort Washington PA)-PBS for 15 min at room temperature to fix cells. After three washes in PBS, slides were incubated in 1% H_2O_2 (H-1009; Sigma Chemical)-PBS 10 min at room temperature, washed three times in PBS, and stained. Slides were permeabilized by incubation with 0.5% saponin (S-2149; Sigma Chemical)-PBS for 10 min at room temperature. Slides were then washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal (T-5125; Sigma). Once the cells were permeabilized, the same buffer was used after this step. According to the protocol of the Vectastain Elite ABC kit

Downloaded from www.chestjournal.org at Kokuritsu-Kokusai-Iryo-Center on March 27, 2006