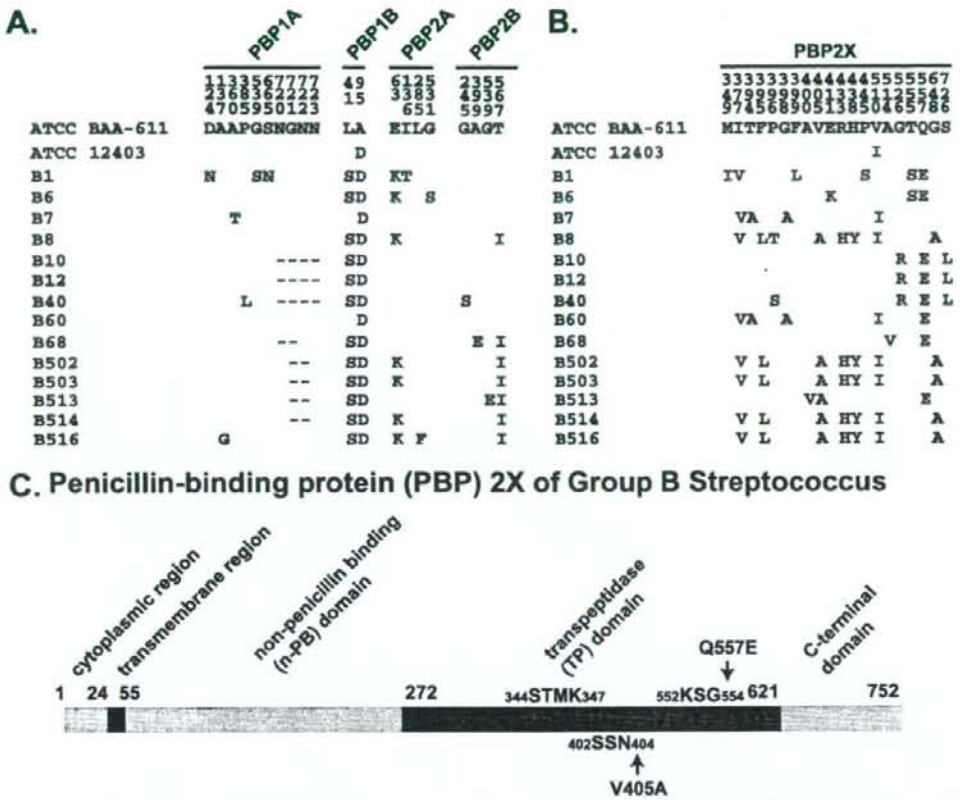

FIG. 2. Deduced amino acid sequences of high-molecular-weight PBPs of control strains (strains ATCC BAA-611 and ATCC 12403) and clinical isolates (isolates B1 to B516). (A and B) Deduced amino acid sequences of high-molecular-weight PBPs (PBP 1A, PBP 1B, PBP 2A, PBP 2B, and PBP 2X). The numbers above the sequences indicate the position number of the amino acid sequence. The blanks and dashes indicate no substitution and deletional change of amino acid, respectively. (C) Pattern diagram of PBP 2X. Common amino acid substitution types V405A and Q557E exist adjacent to active site motifs SSN and KSG, respectively.

G (MICs, ≤0.12 μg/ml) for beta-hemolytic streptococci established by the CLSI. Previously, although several susceptibility test studies have indeed mentioned the existence of β-lactam-insusceptible GBS strains (3, 8, 19), none have intensively identified the GBS strains and investigated the resistance mechanisms on a molecular basis. Therefore, we definitely reidentified the GBS clinical isolates using several different methods, including Gram staining, 16S rRNA gene fingerprinting, biochemical analysis, the CAMP test, and surface antigen analysis. Thus, the present study is the first to confirm the existence of β-lactam-insusceptible strains of GBS (2. 6, 22).

In the present study, we obtained PRGBS candidates through screening using the oxacillin insusceptibility breakpoint of >2 μg/ml. However, the oxacillin MICs for the PRGBS strains were 2 to 8 μg/ml and the difference in the oxacillin MICs between the PRGBS and the penicillin-susceptible GBS strains was not as wide as we had expected. On the other hand, the ceftizoxime MICs for the PRGBS strains ranged from 4 to 128 μg/ml, and the difference in the ceftizoxime MICs for the PRGBS and the penicillin-susceptible GBS strains was much clearer than the difference in the oxacillin MICs. Therefore, we think that, at present, ceftizoxime would be a better agent for use for screening for PRGBS than oxacillin.

Interestingly, all PRGBS strains analyzed in this study were isolated from a nonsterile body site. Indeed, the possibility that PRGBS could lose the ability to cause invasive diseases may not be denied, but PRGBS strains colonizing a nonsterile body site may well translocate to a sterile body site, such as the bloodstream or cerebrospinal fluid. Therefore, it seems very important to screen GBS in clinical specimens taken even from a nonsterile body site, including both the upper respiratory and genital tracts, for reduced penicillin susceptibility.

In the present study, we confirmed through the allelic exchange experiments that the common substitutions found in PBP 2X, Q557E and/or V405A, in the 14 clinically isolated PRGBS strains were the crucial determinants of penicillin G insusceptibility in GBS. In addition, we found a reduced amount of PBP 2X in all PRGBS strains tested. While we consider that the amino acid substitutions in PBP 2X of PRGBS strains are necessary for penicillin insusceptibility in GBS, those amino acid substitutions might make PBP 2X un-

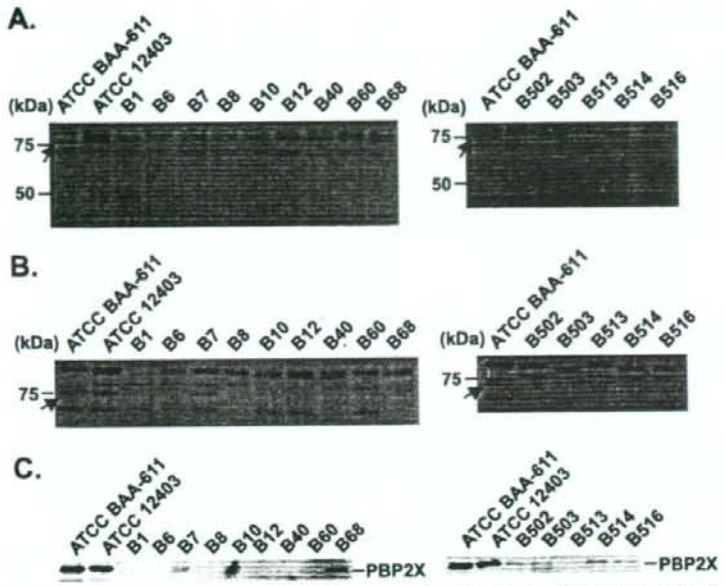

FIG. 3. Visualization of PBPs and Western blotting of PBP 2X from control strains (strains ATCC BAA-611 and ATCC 12403) and clinical isolates (isolates B1 to B516). (A and B) Visualized PBPs. Membrane fractions derived from strains were incubated with fluorescent-conjugated penicillin, followed by SDS-PAGE on a 10% (A) or 6% (B) polyacrylamide gel. Arrows indicate the band corresponding to PBP 2X. (C) Western blotting of PBP 2X. Western blotting analysis was performed with membrane fractions derived from strains and rabbit anti-PBP 2X serum.

stable. Therefore, we assume that the amount of PBP 2X might have been reduced in the PRGBS strains due to the instability of the PBP 2X caused by amino acid substitutions. We assume that the reduction in the amount of PBP 2X is not necessarily responsible for penicillin insusceptibility in PRGBS and that the reduced binding ability of β-lactams to mutated PBP 2X of PRGBS would be a main cause of such penicillin insusceptibility. The reduction in the amount of PBP 2X found in PRGBS might simply be a secondary effect of amino acid substitutions in PBP 2X that might impair the stability of the tertiary structure of the altered PBP 2X molecule. Although the actual molecular mechanisms underlying the penicillin-insusceptible phenotype in GBS should be continuously investigated hereafter, the present study clearly demonstrated that the mutations in the PBP 2X gene are a leading cause of the penicillin-insusceptible phenotype in GBS.

Although the existence of β-lactam-insusceptible strains of GBS had not been confirmed until the present study, the collection of PRGBS strains identified in this study contained clinical isolates stocked from 1995 to 1998. Therefore, we consider that although PRGBS have indeed existed since the 1990s, it has not been confirmed and acknowledged to date. Actually, penicillin insusceptibility in GBS has not been identified thus far in daily clinical laboratory testing due to the absence of criteria for penicillin "resistance" for these microbes. Moreover, it would be difficult to detect PRGBS by routine laboratory testing with penicillin G and ampicillin because the levels of resistance of PRGBS to these agents are not very clear. Hence, the establishment of "resistance" criteria for GBS and the development of a feasible and reliable method for screening for reduced penicillin susceptibility in GBS are warranted.

TABLE 4. Elevation of MICs of nine β-lactams for integrants

Strain	MIC (μg/ml)[a]								
	PEN	PCV	AMP	OXA	CFZ	FEP	CTX	ZOX	MEM
S. pneumoniae ATCC 49619	0.5	0.5	0.12	2	1	0.25	0.12	0.5	0.12
S. agalactiae ATCC 12403	0.06	0.06	0.12	0.25	0.25	0.25	0.06	0.25	0.06
S. agalactiae ATCC BAA-611	0.06	0.06	0.12	0.25	0.12	0.12	0.06	0.25	0.06
ATCC BAA-611 (B12 PBP 2X)	0.5	0.5	0.12	4	2	0.5	0.5	32	0.12
ATCC BAA-611 (B503 PBP 2X)	0.25	0.12	0.25	2	0.5	0.5	0.12	16	0.12
S. agalactiae B12	0.5	0.5	0.12	4	2	1	1	32	0.12
S. agalactiae B503	0.25	0.12	0.25	2	0.5	0.5	0.12	16	0.12

[a] Abbreviations: PEN, penicillin G; PCV, penicillin V; AMP, ampicillin; OXA, oxacillin; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; ZOX, ceftizoxime; MEM, meropemem.

ACKNOWLEDGMENTS

This work was supported by a grant (H18-Shinko-11) from the Ministry of Health, Labor and Welfare, Japan. K.K. is a fellow of the Japan Health Sciences Foundation.

We thank Emmanuelle Maguin and Akihito Wada for providing thermosensitive shuttle vector pG+host6 and β-toxin-producing strain of *S. aureus*, respectively. We are grateful to Kumiko Kai, Yoshie Taki, and Fusako Yokokawa for technical assistance; Miyuki Asano, Hiroyuki Kidokoro, and Yoshiaki Sato for valuable advice regarding the clinical aspects; and Sayaka Takemoto-Kimura for constructive discussion and support.

REFERENCES

1. **American Academy of Pediatrics Committee on Infectious Disease, Committee on Fetus and Newborn.** 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. Pediatrics **99**:489–496.
2. **Baker, C. J.** 2000. Group B streptococcal infections, p. 222–237. *In* D. L. Stevens and E. L. Kaplan (ed.), Streptococcal infections. Clinical aspects, microbiology, and molecular pathogenesis. Oxford University Press, Oxford, England.
3. **Berkowitz, K., J. A. Regan, and E. Greenberg.** 1990. Antibiotic resistance patterns of group B streptococci in pregnant women. J. Clin. Microbiol. **28**:5–7.
4. **Biswas, I., A. Gruss, S. D. Ehrlich, and E. Maguin.** 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. **175**:3628–3635.
5. **Centers for Disease Control and Prevention.** 2002. Prevention of perinatal group B streptococcal disease. MMWR Recommend. Rep. **51**(RR-11):1–22.
6. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial susceptibility testing, p. 138–140; 16th informational supplement M100-S16, vol. 26. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Committee on Obstetric Practice.** 1996. Prevention of early-onset group B streptococcal disease in newborns. American College of Obstetricians and Gynecologists, Washington, DC.
8. **De Azavedo, J. C. S., M. Mcgavin, C. Duncan, D. E. Low, and A. McGeer.** 2001. Prevalence and mechanisms of macrolide resistance in invasive and noninvasive group B streptococcus isolates from Ontario, Canada. Antimicrob. Agents Chemother. **45**:3504–3508.
9. **Edwards, M. S., and C. J. Baker.** 2005. Group B streptococcal infections in elderly adults. Clin. Infect. Dis. **41**:839–847.
10. **Farley, M. M., R. C. Harvey, T. Stull, J. D. Smith, A. Schuchat, J. D. Wenger, and D. S. Stephens.** 1993. A population-based assessment of invasive disease due to group B streptococcus in nonpregnant adults. N. Engl. J. Med. **328**:1807–1811.
11. **Farley, M. M.** 2001. Group B streptococcal disease in nonpregnant adults. Clin. Infect. Dis. **33**:556–561.
12. **Framson, P. E., A. Nittayajarn, J. Merry, P. Youngman, and C. E. Rubens.** 1997. New genetic techniques for group B streptococci: high-efficiency transformation, maintenance of temperature-sensitive pWV01 plasmids, and mutagenesis with Tn*917*. Appl. Environ. Microbiol. **63**:3539–3547.
13. **Hansen, S. M., N. Uldbjerg, M. Kilian, and U. B. Skov Sorensen.** 2004. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. J. Clin. Microbiol. **42**:83–89.
14. **Heath, P. T., G. Balfour, A. M. Weisner, A. Efstratiou, T. L. Lamagni, H. Tighe, L. A. O'Connell, M. Cafferkey, N. Q. Verlander, A. Nicoll, A. C. McCartney, and PHLS Group B Streptococcus Working Group.** 2004. Group B streptococcal disease in UK and Irish infants younger than 90 days. Lancet **363**:292–294.
15. **Jackson, L. A., R. Hilsdon, M. M. Farley, L. H. Harrison, A. L. Reingold, B. D. Plikaytis, J. D. Wenger, and A. Schuchat.** 1995. Risk factors for group B streptococcal disease in adults. Ann. Intern. Med. **123**:415–420.
16. **Johri, A. K., L. C. Paoletti, P. Glaser, M. Dua, P. K. Sharma, G. Grandi, and R. Rappuoli.** 2006. Group B streptococcus: global incidence and vaccine development. Nat. Rev. Microbiol. **4**:932–942.
17. **Kimura, K., J.-I. Wachino, H. Kurokawa, S. Suzuki, K. Yamane, N. Shibata, and Y. Arakawa.** 2006. Emergence of penicillin-resistant group B streptococci, abstr. C2-1286. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC.
18. **Lu, J.-J., C.-L. Perng, S.-Y. Lee, and C.-C. Wan.** 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38**:2076–2080.
19. **Morikawa, Y., M. Kitazato, C. Katsukawa, and A. Tamaru.** 2003. Prevalence of cefotaxime resistance in group B streptococcus isolates from Osaka, Japan. J. Infect. Chemother. **9**:131–133.
20. **Schrag, S. J., S. Zywicki, M. M. Farley, A. L. Reingold, L. H. Harrison, L. B. Lefkowitz, J. L. Hadler, R. Danila, P. R. Cieslak, and A. Schuchat.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. N. Engl. J. Med. **342**:15–20.
21. **Schuchat, A.** 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin. Microbiol. Rev. **11**:497–513.
22. **Schuchat, A.** 1999. Group B streptococcus. Lancet **353**:51–56.
23. **Stoll, B. J., N. Hansen, A. A. Fanaroff, L. L. Wright, W. A. Carlo, R. A. Ehrenkranz, J. A. Lemons, E. F. Donovan, A. R. Stark, J. E. Tyson, W. Oh, C. R. Bauer, S. B. Korones, S. Shankaran, A. R. Laptook, D. K. Stevenson, L. A. Papile, and W. K. Poole.** 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N. Engl. J. Med. **347**:240–247.
24. **Van Asselt, G. J., G. de Kort, and J. A. M. van de Klundert.** 1995. Differences in penicillin-binding protein patterns of penicillin tolerant and non-tolerant group A streptococci. J. Antimicrob. Chemother. **35**:67–74.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2008, p. 2287–2288 Vol. 52, No. 6
0066-4804/08/$08.00+0 doi:10.1128/AAC.00022-08

Nomenclature of Plasmid-Mediated 16S rRNA Methylases Responsible for Panaminoglycoside Resistance▽

Production of 16S rRNA methylase has recently drawn attention as a novel aminoglycoside resistance mechanism in pathogenic gram-negative bacteria (1). It confers very-high-level resistance to all aminoglycosides that are currently available for parenteral formulation. Six distinct genes, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *armA*, and *npmA*, encoding their respective enzymes have been identified in clinical and veterinary strains from various geographic areas, including East Asia, Europe, and the Americas, since 2003 (1, 10). NpmA is the only enzyme among them that methylates residue A1408, whereas the others methylate residue G1405, both within the aminoacyl site (A site) of the 16S rRNA (7, 10). All six genes are confirmed to be or are likely to locate on plasmids (3, 4, 10, 11, 12, 14). Recent findings also indicate that some of these genes are capable of crossing the barrier between glucose-fermenting and nonfermenting species. For instance, *armA* has been identified in both members of the family *Enterobacteriaceae* and in *Acinetobacter baumannii* (5, 13), and *rmtD* has been identified in *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (unpublished data). We will likely see an increasing number of reports about this resistance mechanism, including identification of genes encoding new 16S rRNA methylases.

Historically, the nomenclature of genes and enzymes for many resistance mechanisms has become complicated and nonsystematic (6). An extreme example is that of aminoglycoside acetyltransferases, where new gene names are arbitrarily assigned from one of the two coexisting nomenclature systems (9). The situation is somewhat better with β-lactamases and macrolide resistance genes, due to a registry and guidelines, respectively (http://www.lahey.org/Studies/) (8). To prevent confusion over the nomenclature of 16S rRNA methylases, we would like to propose practical rules for the nomenclature of these enzymes, which shall apply to any relevant enzymes to be identified in the future.

Currently, the highest and lowest identities of amino acid sequences among the G1405 16S rRNA methylases are 81.7% between RmtA and RmtB and 25.8% between ArmA and RmtD, respectively (2, 3). On the other hand, identities lower than 10% are observed between the G1405 16S rRNA methylases and the NpmA that methylates A1408 (10) (Table). Thus, we propose the following rules. A gene that has an amino acid identity greater than 95% with the closest known 16S rRNA methylase gene will be assigned a variant number starting from two in the order of the dates on which the sequences are deposited in the GenBank/EBML/DDBJ, e.g., *rmtA2* and then *rmtA3*, analogous to the nomenclature of the *qnr* genes. A gene that has between 50 and 95% amino acid identity with the closest known 16S rRNA methylase gene will be assigned a new alphabet letter according to the closest existing gene name, e.g., *rmtE*, *rmtF*, *armB*, *or armC*, provided that the gene is shown to confer a consistent aminoglycoside resistance profile. A gene that has either an amino acid identity of less than 50% with the closest known 16S rRNA methylase gene or that is proven to methylate a new residue of 16S rRNA may be assigned a brand new gene name, like *npmA*, contingent upon demonstration of 16S rRNA methylation activity of the gene product and attributable resistant phenotype. Data regarding 16S rRNA methylase genes in pathogenic bacteria will be accumulated and provided at the following website http://www.nih.go.jp/niid/16s_database/index.html.

TABLE 1. Identity of amino acid residues among the sequences of plasmid-mediated 16S rRNA methylases

Methylase	Amino acid sequence identity (%)[a]					
	RmtA	RmtB	RmtC	RmtD	ArmA	NpmA
RmtA	100	81.7	27.7	41.2	29.2	<10
RmtB		100	29.5	41.3	28.9	<10
RmtC			100	26.0	27.8	<10
RmtD				100	25.8	<10
ArmA					100	<10
NpmA						100

[a] Identities were calculated by GENETYX, Macintosh version 14.0.1 (SDC Co., Ltd., Tokyo, Japan).

Studies of plasmid-mediated 16S rRNA methylases identified among pathogenic microbes were supported by a grant (H18-Shinkou-011) from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

1. **Doi, Y., and Y. Arakawa.** 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. **45**:88–94.
2. **Doi, Y., D. de Oliveira Garcia, J. Adams, and D. L. Paterson.** 2007. Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrob. Agents Chemother. **51**:852–856.
3. **Doi, Y., K. Yokoyama, K. Yamane, J. Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. **48**:491–496.
4. **Galimand, M., P. Courvalin, and T. Lambert.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. **47**:2565–2571.
5. **Galimand, M., S. Sabtcheva, P. Courvalin, and T. Lambert.** 2005. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrob. Agents Chemother. **49**:2949–2953.
6. **Hall, R., and S. Partridge.** 2003. Unambiguous numbering of antibiotic resistance genes. Antimicrob. Agents Chemother. **47**:3998–3999.
7. **Liou, G. F., S. Yoshizawa, P. Courvalin, and M. Galimand.** 2006. Aminoglycoside resistance by *ArmA*-mediated ribosomal 16S methylation in human bacterial pathogens. J. Mol. Biol. **359**:358–364.
8. **Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppala.** 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. **43**:2823–2830.
9. **Vanhoof, R., E. Hannecart-Pokorni, and J. Content.** 1998. Nomenclature of genes encoding aminoglycoside-modifying enzymes. Antimicrob. Agents Chemother. **42**:483.
10. **Wachino, J., K. Shibayama, H. Kurokawa, K. Kimura, K. Yamane, S. Suzuki, N. Shibata, Y. Ike, and Y. Arakawa.** 2007. Novel plasmid-mediated 16S rRNA m^{1}A1408 methyltransferase, NpmA, found in a clinically isolated *Escherichia coli* strain resistant to structurally diverse aminoglycosides. Antimicrob. Agents Chemother. **51**:4401–4409.
11. **Wachino, J., K. Yamane, K. Shibayama, H. Kurokawa, N. Shibata, S. Suzuki, Y. Doi, K. Kimura, Y. Ike, and Y. Arakawa.** 2006. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob. Agents Chemother. **50**:178–184.
12. **Yamane, K., F. Rossi, M. G. Barberino, J. M. Adams-Haduch, Y. Doi, and D. L. Paterson.** 2008. 16S ribosomal RNA methylase RmtD produced by *Klebsiella pneumoniae* in Brazil. J. Antimicrob. Chemother. **61**:746–747.

13. **Yamane, K., J. Wachino, Y. Doi, H. Kurokawa, and Y. Arakawa.** 2005. Global spread of multiple aminoglycoside resistance genes. Emerg. Infect. Dis. **11**:951–953.

14. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362**:1888–1893.

Yohei Doi
Division of Infectious Diseases
University of Pittsburgh Medical Center
Pittsburgh, Pennsylvania

Jun-ichi Wachino
Yoshichika Arakawa*
Department of Bacterial Pathogenesis and Infection Control
National Institute of Infectious Diseases
4-7-1 Gakuen
Musashi-Murayama, Tokyo 208-0011
Japan

*Phone: 81-42-561-0771, ext. 500
Fax: 81-42-561-7173
E-mail: yarakawa@nih.go.jp

[9] Published ahead of print on 31 March 2008.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2008, p. 4258–4267 Vol. 52, No. 12
0066-4804/08/$08.00+0 doi:10.1128/AAC.00596-08

Genetic Heterogeneity in *pbp* Genes among Clinically Isolated Group B Streptococci with Reduced Penicillin Susceptibility▽

Noriyuki Nagano,[1,2] Yukiko Nagano,[1] Kouji Kimura,[1] Kiyoko Tamai[1,3]
Hideji Yanagisawa,[3] and Yoshichika Arakawa[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] Medical Microbiology Laboratory, Funabashi Municipal Medical Center, Chiba,[2] and Miroku Medical Laboratory Co., Ltd., Nagano,[3] Japan

Received 7 May 2008/Returned for modification 11 July 2008/Accepted 12 September 2008

The recent emergence of group B streptococcal isolates exhibiting increased penicillin MICs at the Funabashi Municipal Medical Center and other hospitals in Japan prompted a comparative analysis of the penicillin-binding proteins (PBPs) from those strains with the PBPs from penicillin-susceptible strains comprising four neonatal invasive strains isolated from 1976 to 1988 and two recent isolates. The PBP sequences of the penicillin-susceptible strains were highly conserved, irrespective of their isolation date. Of six strains with reduced susceptibility to penicillin (penicillin MICs, 0.25 to 0.5 μg/ml), strains R1, R2, R5, and R6 shared a unique set of five amino acid substitutions, including V405A adjacent to the $_{402}$SSN$_{404}$ motif in PBP 2X and one in PBP 2B. The remaining two strains, R3 and R4, shared several substitutions, including Q557E adjacent to the $_{552}$KSG$_{554}$ motif in PBP 2X, in addition to the substitutions in PBP 2B, which are commonly found among penicillin-insusceptible strains. Strains R7 and R8, which had a penicillin MIC of 1 μg/ml, shared a unique set of eight amino acid substitutions (two in PBP 2X; two in PBP 2B, including G613R adjacent to the $_{614}$KTG$_{616}$ motif; three in PBP 1A; and one in PBP 2A), and the Q557E substitution in PBP 2X was common to R3 and R4. The binding of Bocillin FL was reduced or not detected in some PBPs, including PBP 2X of penicillin-insusceptible strains, but no significant reduction in the level of *pbp2x* transcription was found in such strains. The results of phylogenetic comparative analyses imply the absence of epidemic penicillin-insusceptible strains, and several genetic lineages of penicillin-insusceptible strains have been independently emerging through the accumulation of mutations in their *pbp* genes, especially in *pbp2x*.

Group B streptococcus (GBS), which composes a part of the normal vaginal flora in 10 to 35% of healthy women, is one of the most important causes of neonatal sepsis and meningitis (29, 31). GBS also causes cutaneous and invasive infections in pregnant women and nonpregnant adults, including elderly individuals and immunocompromised patients (8, 32). Penicillin is the first-line antibiotic for GBS disease therapy as well as for intrapartum chemoprophylaxis. Resistance to this agent has so far not been reported among GBS isolates, while survey studies have shown a high prevalence of resistance to macrolides, followed by fluoroquinolones, among invasive and noninvasive isolates (3, 9, 14, 18, 24, 40). However, a trend toward an increase in the MICs of β-lactam antibiotics has occasionally been noted in recent reports (7, 16, 22), although no detailed analysis of the molecular mechanism associated with the loss of susceptibility had been conducted before the recently described studies (6, 19). This phenomenon elicits concern for the future prevalence of GBS strains with increased resistance to β-lactams, as has been noted for *Streptococcus pneumoniae*, in which penicillin-intermediate resistant pneumococcal strains were reported in the late 1960s (1, 13), followed by the reporting of more highly resistant strains (MICs, ≥2 μg/ml) whose drug resistance expanded not only to β-lactams but also to other antimicrobial agents in the late 1970s (2, 20).

Penicillin resistance in gram-positive organisms, including *S. pneumoniae*, is essentially due to the production of altered, low-affinity target enzymes, penicillin-binding proteins (PBPs) that catalyze the terminal stage of bacterial cell wall peptidoglycan synthesis. In PBPs, three conserved motifs, SXXK, SXN, and KT(S)G, commonly found in transpeptidase domains form the catalytic center; and alterations within or adjacent to these motifs are associated with their reduced affinity for β-lactams (10, 34). As for *S. pneumoniae*, in particular, substitutions in the amino acid residues of PBP 2B, PBP 2X, and PBP 1A, which are among the five high-molecular-weight PBPs, confer the development of β-lactam resistance in this organism; altered PBP 2B and PBP 2X enzymes confer low-level resistance to piperacillin and cefotaxime, respectively (12), and an additional alteration in PBP 1A confers high-level resistance to β-lactams (23, 33).

We recently encountered several GBS strains insusceptible to penicillin and other β-lactams at the Funabashi Municipal Medical Center and other hospitals in Japan. The emergence of resistance to those drugs in GBS would have significant clinical implications, which prompted us to seek to gain an in-depth understanding of the isolates with reduced susceptibility to penicillins. In a very recent work, Kimura et al. reported that altered PBP 2X makes a major contribution to the reduction of GBS susceptibility to β-lactams (19). The present study investigated the association between the amino acid substitutions found in high-molecular-weight PBPs, the affinities of PBPs to penicillins, and the levels of increased penicillin

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

▽ Published ahead of print on 22 September 2008.

MICs for isolates with reduced susceptibilities to penicillins. Our analysis is unique in that it was supported by the inclusion of a group of penicillin-susceptible strains comprising four neonatal invasive strains isolated from 1976 to 1988 and two recently isolated strains for phylogenetic comparative analysis.

MATERIALS AND METHODS

Bacterial strains. For PBP analysis, eight GBS clinical strains (strains R1 to R8) with penicillin MICs of 0.25 to 1 μg/ml recovered during 2003 and 2004 were selected from our bacterial culture collection. Included were penicillin-susceptible strains, four of which (strains N1 to N4) were from neonatal invasive infections recovered from 1976 to 1988, and two of which, strains C1 and C2, were recovered from vaginal and stool specimens obtained in 2004. The origins of these strains are presented in Table 1.

The GBS strains were grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD) at 37°C.

The GBS clinical strains were serotyped with antisera (Denka Seiken, Tokyo, Japan) to the type-specific capsular polysaccharides Ia, Ib, II, III, IV, V, VI, VIII, and 7271. Strains 2603 V/R (ATCC BAA-611; GenBank accession number NC 004116) and NEM316 (ATCC 12403; GenBank accession number NC 004368) were used as reference strains for comparative characterization.

No β-lactamase activity was detected by the acidimetric method in any of the GBS strains subjected to this study.

Antimicrobial susceptibility testing. MICs were determined by a broth microdilution method with a MicroScan MICroFAST panel type 3J system (Dade Behring Inc., Tokyo, Japan) by following the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) (4, 5). MIC determinations were performed five times for each strain to ensure the reproducibility of the MICs by using quality control strain *S. pneumoniae* ATCC 49619.

Analysis of *pbp* gene sequences. For all 14 clinical strains, five *pbp* genes encoding high-molecular-weight PBPs 1A, 1B, 2A, 2B, and 2X identified in the strain 2603 V/R genome available from the GenBank database were sequenced. Genomic DNAs were extracted from each strain with a Wizard genomic DNA purification kit (Promega, Madison, WI) and 20 U of mutanolysin (27). The entire coding regions of the *pbp1a*, *pbp1b*, *pbp2a*, *pbp2b*, and *pbp2x* genes were amplified with primer pairs f1 and r1, designed on the basis of the sequence of the corresponding gene of strain 2603 V/R, as listed in Table 2. PCRs were carried out with Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan) and the following parameters: initial denaturation at 98°C for 1 min, denaturation at 98°C for 10 s, primer annealing at 55°C for 1 min, and extension at 72°C for 2.5 min, repeated for 30 cycles, and a final extension at 72°C for 7 min. The amplified DNA fragments were purified with a Wizard SV Gel and PCR cleanup system (Promega). For each *pbp* gene, several internal forward and reverse sequencing primers were designed on the basis of the published sequence of strain 2603 V/R (Table 2) and were used for the sequencing reactions to provide complete coverage of the sequences as well as to ensure the accuracy of the sequence data. Sequencing of both strands was performed with a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism model 3100 genetic analyzer (Applied Biosystems).

The sequences obtained were assembled into contigs with BioEdit (version 5.0.9) software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analyses for the sequences determined were conducted with the MEGA program (version 3.1) (21). The sequences were aligned with Clustal W software (37), and distance-based analyses were conducted by using Kimura's two-parameter model distance matrices at the nucleotide level. Phylogenetic trees were constructed by the neighbor-joining method in the MEGA program. A bootstrap analysis (500 repeats) was performed to evaluate the topology of the phylogenetic tree.

Membrane preparation and labeling of PBPs with fluorescent penicillin. Membrane proteins were prepared as described by van Asselt et al. (39), except that the cells were disrupted by passage through a French press twice at a pressure of 120 MPa, and aliquots of the membrane proteins were stored at −80°C at a concentration of 10 mg/ml until use. The quantity of protein was determined with a BCA protein assay kit (bicinchoninic acid method; Pierce, Rockville, IL).

For detection of PBPs, 400 μg of the membrane proteins was mixed with 12.5 μM Bocillin FL (Molecular Probes, Inc., Eugene, OR), incubated at 37°C for 30 min, and denatured by adding 50 μl of 3× Laemmli sample buffer and heating to 100°C for 3 min. The labeled PBPs (40 μg protein/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5 or 10% gels and were visualized by fluorography with an LAS-3000 multicolor image analyzer (excitation at 460 nm; Fujifilm, Tokyo, Japan).

Immunodetection of PBP 2X. To identify PBP 2X, immunoblotting was performed with a polyclonal antibody. Anti-PBP 2X was developed in rabbits by using as an immunogen a synthetic peptide (GKVTYEKDRSGNVL) corresponding to amino acids 237 to 250 of PBP 2X from *S. agalactiae* 2603 V/R conjugated to keyhole limpet hemocyanin via an N-terminal added cysteine residue (19).

The membrane proteins were separated by SDS-PAGE as described above and electroblotted onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). The immobilized proteins were probed with the anti-PBP 2X antibody (1:1,000) and a goat anti-rabbit immunoglobulin G horseradish peroxidase antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were detected by chemilumigraphy with an ECL Western blotting detection system (GE Healthcare) and an LAS-3000 multicolor image analyzer.

Real-time RT-PCR analysis of *pbp2x* expression. The GBS strains were grown to an optical density at 600 nm of 0.3, and total RNA was isolated by using the RNAprotect bacterial reagent and an RNeasy mini kit (Qiagen GmbH, Hilden, Germany), as described previously (26), and the integrity was checked by agarose gel electrophoresis. The RNA was quantified spectrophotometrically and stored in aliquots at −70°C. DNase-treated RNA was reverse transcribed with a PrimeScript reverse transcription (RT) reagent kit (Takara), according to the instructions of the manufacturer, with 5 μM random 6-mers. Real-time PCR analysis was performed in triplicate on the SmartCycler system (Cepheid, Sunnyvale, CA) with 2 μl of cDNA (200 pg to 80 ng), SYBR Premix Ex *Taq* (Takara), and 0.2 μM each of the specific primers (Table 2), according to the manufacturer's protocol. The levels of the target transcripts were normalized to those of the internal reference gene, the gene for prolyl-tRNA synthetase (*pros*) (11), in each sample. The values for the *pbp2x* transcripts of strain 2603 V/R were normalized to 1, and other data were calculated relative to this value. For comparison between penicillin-insusceptible and -susceptible strains, statistical analysis of variance, followed by the unpaired Student *t* test, was performed. A P value of <0.05 was considered statistically significant.

Chromosomal DNA restriction profiles. Molecular analysis of the chromosomal DNAs was performed as previously described by Nagano et al. (25), with some modifications. Briefly, chromosomal DNAs were extracted with mutanolysin in agarose gel plugs and were then incubated overnight at 30°C with 20 U of either SmaI (Takara) or ApaI (Takara). Bacteriophage lambda DNA ladders (48.5 kb to 1 Mb; Takara) were used as molecular size markers.

Epidemiological study. Epidemiological analysis of the MIC distributions was performed with 442 clinical isolates collected from various regions in Japan between March 2005 and February 2006 by the Miroku Medical Laboratory.

Nucleotide sequence accession numbers. The nucleotide sequences of the *pbp* genes from all the GBS strains tested in this study were deposited in the EMBL/GenBank through DDBJ under accession numbers AB368300 to AB368369.

RESULTS

MICs. The MICs of several antimicrobial agents for *S. pneumoniae* ATCC 49619 were all within the quality control ranges defined by the CLSI (5), and reproducible MIC results were obtained for the GBS strains tested against all antimicrobials (Table 1). Strains N1 to N4, C1, C2, 2603 V/R, and NEM316 were all susceptible to β-lactams: the penicillin MICs were 0.06 μg/ml, the ampicillin MICs ranged from 0.12 to 0.25 μg/ml, the cefotaxime MICs ranged from ≤0.06 to 0.12 μg/ml, and the cefepime MICs were ≤0.5 μg/ml. Strains R1 to R6 were all insusceptible to penicillin (MICs, 0.25 to 0.5 μg/ml), whereas the ampicillin MICs were 0.25 μg/ml for R3 and R4 and 0.5 μg/ml (insusceptible) for R1, R2, R5, and R6. The cefepime MICs were ≤0.5 μg/ml for R3 and 1 μg/ml (insusceptible) for R1, R2, and R4 to R6. These six strains were susceptible to cefotaxime (MIC, 0.5 μg/ml), but this MIC was higher than the MICs for strains N1 to N4, C1, C2, 2603 V/R, and NEM316. Strains R7 and R8 were insusceptible to these β-lactams; the MICs of penicillin, ampicillin, cefotaxime, and cefepime were 1 μg/ml, 0.5 μg/ml, 1 μg/ml, and 1 to 2 μg/ml, respectively. Increased MICs of the other β-lactams, especially those of cefotiam and cefozopran, were also noted in strains R1 to R8.

TABLE 1. Origins and MICs of GBS isolates[a]

Strain	Strain or geographic origin in Japan	Ward(s)	Patient			Specimen	Date of isolation (mo/yr)	Serotype	MIC (μg/ml)												
			Sex, age	Underlying disease(s)	Prior therapy				PEN	AMC	CTX	CTM	CFM	FEP	CZOP	CDN	MEM	CLR	ERY	CLI	LVX	TET
2603 V/R	ATCC BAA-611							V	0.06	0.12	≤0.06	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4
NEM 316	ATCC 12403							III	0.06	0.12	≤0.06	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	≤0.5
N1	Hokkaido (FMC collection)	Pediatrics	—, 50 days	Meningitis	None	CSF	1976	Ia	0.06	0.12	0.12	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	1	>4
N2	Hokkaido (FMC collection)	Pediatrics	—, 90 days	Meningitis	None	CSF	1988	Ib	0.06	0.25	0.12	≤0.5	1	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	≤0.5
N3	FMC Funabashi, Chiba	Pediatrics	M, 90 days	Meningitis	None	CSF	1983	III	0.06	0.12	0.12	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4
N4	Hokkaido (FMC collection)	Pediatrics	—, 1 day	Sepsis, pneumonia	None	BL	1985	VI	0.06	0.12	≤0.06	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4
C1	FMC Funabashi, Chiba	Gynecology	F, 27 yr	None	None	VA	03/2004	V	0.06	0.12	≤0.06	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4
C2	FMC Funabashi, Chiba	Neurosurgery	F, 75 yr	Diabetes mellitus	LVFX, CEZ	ST	11/2004	Ib	0.06	0.25	0.12	≤0.5	0.5	≤0.5	0.12	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	>8	≤0.5
R1	FMC Funabashi, Chiba	Neurosurgery	M, 74 yr	Acute subdural hematoma, brain contusion	TOB, CAZ	TTA	10/2003	VI	0.5	0.5	0.5	4	>1	1	0.5	0.25	0.25	≤0.12	≤0.12	≤0.12	8	>4
R2	FMC Funabashi, Chiba	Neurosurgery	M, 55 yr	Cerebral infarction	CEZ, GM CAZ	TTA	11/2003	VI	0.5	0.5	0.5	4	>1	1	1	0.25	0.5	≤0.12	≤0.12	≤0.12	8	>4
R3	FMC Funabashi, Chiba	Urology, ophthalmology	M, 84 yr	Multiple cerebral infarction	None	CS	03/2004	Ib	0.5	0.25	0.5	4	>1	≤0.5	1	0.25	0.25	≤0.12	≤0.12	≤0.12	>8	≤0.5
R4	Hospital A Choshi, Chiba	Internal medicine	F, 89 yr	—	—	SP	12/2004	VI	0.5	0.25	0.5	4	>1	1	0.5	0.25	0.25	>2	>2	>1	2	>4
R5	Hospital B Kodaira, Tokyo	Internal medicine	F, 96 yr	—	—	PHA	12/2003	VI	0.25	0.5	0.5	2	>1	1	0.5	0.25	0.25	≤0.12	≤0.12	≤0.12	4	>4
R6	Hospital C Tachikawa, Tokyo	Internal medicine	F, adult	—	—	SP	09/2004	VI	0.5	0.5	0.5	4	>1	1	0.5	0.25	0.5	≤0.12	≤0.12	≤0.12	4	>4
R7	Hospital D Ichinoseki, Iwate	Internal medicine	M, adult	—	—	SP	09/2004	Ia	1	0.5	1	>4	>1	2	2	0.5	0.25	2	>2	≤0.12	>8	>4
R8	Hospital D Ichinoseki, Iwate	Internal medicine	F, 80 yr	—	—	SP	01/2004	NT	1	0.5	1	>4	>1	1	1	0.5	0.25	1	>2	≤0.12	>8	>4

[a] PEN, penicillin; AMC, ampicillin; CTX, cefotaxime; CTM, cefotiam; CFM, cefixime; FEP, cefepime; CZOP, cefozopran; CDN, cefditoren; LVFX, levofloxacin; MEM, meropenem; CLR, clarithromycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; LVX, levofloxacin; CEZ, cefazolin; CAZ, ceftazidime; GM, gentamicin; FMC, Funabashi Medical Center; M, male; F, female; TTA, transtracheal aspirate; VA, vaginal discharge; CS, conjunctival sac discharge; ST, stool; PHA, pharyngeal swab; SP, sputum; CSF, cerebrospinal fluid; BL, blood; NT, nontypeable; —, unknown.

TABLE 2. Oligonucleotide primers

Procedure(s) and target gene	Primer name	Sequence[a]	Amplicon size (bp)
PCR and sequencing			
pbp1a PCR	f1	5′-CGGAATTCATGGGATTTATTATCTTAGCTA-3′	2,209
	r1	5′-ACGTCGACTTAATTACCGTTAGGTACTGTA-3′	
pbp1a sequencing primers	f1b	5′-ACACCAAAGAAGAAATTCTTAC-3′	
	f2	5′-TAAAGCAAAAATCTACTTATCC-3′	
	f2b	5′-GTAGTGAGAAAATGGCAGCGGC-3′	
	f3	5′-GCCTACATGATGACGGATATGC-3′	
	f3b	5′-CAAAATTCTGGACAGTCAAGTC-3′	
	r2	5′-TCCAATCTGCACTGTATCCGCC-3′	
	r3	5′-TAGCTGCTTTAGTACCAGTACC-3′	
	r4	5′-CAGCGGCTTCAAGTGCTCTGAC-3′	
	r5	5′-TGACTTTACCATTAGTCGCATC-3′	
	r6	5′-TTTTATCTTGATACATCTGCTG-3′	
pbp1b PCR	f1	5′-CGGAATTCATGTTTAAAGGTAATAAGAAGT-3′	2,314
	r1	5′-ACGTCGACTTATCGTTTTCCACCCAAAGTA-3′	
pbp1b sequencing primers	f1b	5′-GGTTTGGAGAGAGTAGCGG-3′	
	f2	5′-CTATTGTATATTCTCCTTATAC-3′	
	f2b	5′-GTATACTATTAAAACTACTATC-3′	
	f3	5′-TGATGTAAAAAACTATATGGAG-3′	
	f3b	5′-CCTGTCCGTGTCTTTTCGAAAG-3′	
	r2	5′-GTGTAGAAAGCATCAACCAAAC-3′	
	r3	5′-GAGCAACTGACGTATCAATACC-3′	
	r4	5′-GATCAATAGCAATTCCGTAAGG-3′	
	r5	5′-TTTTTAAATCATGCTCTGAAAC-3′	
	r6	5′-GTAAACCTGCAAGGAAAGCTGC-3′	
pbp2a PCR	f1	5′-CGGGATCCATGAAATTATTTGATAAGTTTA-3′	2,338
	r1	5′-ACGTCGACCTATCTAAAGTAGTCCTTTAGA-3′	
pbp2a sequencing primers	f1b	5′-TGCTCTAAAAACAACCACCACC-3′	
	f2	5′-ATCTTAATAACTCTTATTTTGG-3′	
	f2b	5′-GGTATGAAAAATAGATTAGCAG-3′	
	f3	5′-TCCTGCTGTTTATACTTTAGAC-3′	
	f3b	5′-ACTCGAATTGAGACAGCTAATG-3′	
	r2	5′-CTGTCAAATAATGGTGTTTATC-3′	
	r3	5′-ATGAGCGCGATGCATTATACCG-3′	
	r4	5′-GTTCTTTATCTATTGACCATCC-3′	
	r5	5′-TATAGCCATTATTGACAATATC-3′	
	r6	5′-TCAAATTAGCAGCACTGGTTCC-3′	
pbp2b PCR	f1	5′-CGGAATTCATGTTGAATCGTAAAAAAAGGT-3′	2,062
	r1	5′-ACGTCGACTTATTGTCCTGTGAACTGTGAA-3′	
pbp2b sequencing primers	f1b	5′-TTCATCTCAGTCTATCAAAGAG-3′	
	f2	5′-CAACTCTAATGGAATCGTTCGG-3′	
	f2b	5′-CTATTTCTACAGAAAAGGCAGG-3′	
	f3	5′-AGAAAGTATCTTGAAACAATAC-3′	
	f3b	5′-TGGACAAACAGTTTCTACCTAC-3′	
	r2	5′-CTATCTTATTTAGTGTTTTAGG-3′	
	r3	5′-GATAGCCTCGATCAGTTAAAGC-3′	
	r4	5′-CATGATCATTTTTCAGACCAGC-3′	
	r5	5′-CTCGGTCATTCAGTGAATAGCC-3′	
	r6	5′-TAGCGCTCACTGGAACTGCAGC-3′	
pbp2x PCR	f1	5′-CGGAATTCGTGACTTTTTTTAAAAAGCTAA-3′	2,275
	r1	5′-ACGTCGACTTAATCTCCTATTGTAATTTTG-3′	
pbp2x sequencing primers	f1b	5′-AACTATACGACAGCTACAGGTC-3′	
	f2	5′-GTAGTGGGAATGTTCTTTTAGG-3′	
	f2b	5′-TCTAAGCATTTTAACTCTACTG-3′	
	f3	5′-AAGAAGCAGCTAGTAAAACACG-3′	
	f3b	5′-GAAAATCCAGGTCATGTAGCGG-3′	
	r2	5′-GAACCAGATTACGACGTAATTC-3′	
	r3	5′-CAGATTTTACTGCAACTGATTG-3′	
	r4	5′-ATGAGCTCATAGCGATAGTTAC-3′	
	r5	5′-TTGCAGAGGCTAGAGTCATTAC-3′	
	r6	5′-CCGCCCTACGTTCTGTTGTTGC-3′	
	r7	5′-AAGACAATCCTGAACCTGAACTTCC-3′	
	r8	5′-TATCTGTACCAACGATGATGAC-3′	
Real-time RT-PCR			
pbp2x	f1	5′-ACCGGGATTGAATGACTCAG-3′	109
	r1	5′-TGGCTGAACCAGATTACGAC-3′	
pros	f1	5′-AATGCCAAGTGATGCTCAGG-3′	84
	r1	5′-GCATAGATTCCAGCCGAAAC-3′	

[a] Restriction sites are underlined.

TABLE 3. Nucleotide mutations in *pbp* genes[a]

Strain	*pbp2x*		*pbp2b*		*pbp1a*		*pbp2a*		*pbp1b*	
	Total	Ns	Total	Ns	Total	Ns	Total	Ns	Total	Ns
N1	0	0	1	1	0	0	10	1	3	1
N2	0	0	0	0	0	0	10	1	3	1
N3	2	1	4	1	7	2	13	3	0	0
N4	0	0	0	0	0	0	10	1	3	1
C1	0	0	0	0	0	0	10	1	3	1
C2	0	0	0	0	0	0	10	1	3	1
R1	11	5	1	1	0	0	10	1	3	1
R2	11	5	1	1	0	0	10	1	3	1
R3	3	2	2	2	1	0	2	0	3	1
R4	2	1	2	2	0	0	3	1	3	1
R5	10	5	1	1	0	0	10	1	3	1
R6	11	6	1	1	0	0	11	2	3	1
R7	9	4	6	2	12	4	1	1	0	0
R8	9	4	6	2	12	4	1	1	0	0

[a] Total, total number of nucleotide substitutions compared to the sequences od *S. agalactiae* 2603 V/R and NEM316; Ns, number of nonsynonymous substitutions.

A trend toward higher MICs of levofloxacin was found for the recently isolated strains.

Nucleotide and deduced amino acid sequences of *pbp* genes. DNA sequencing of the *pbp* genes revealed that the *pbp2x* and *pbp2b* genes of penicillin-insusceptible strains possessed many nucleotide mutations compared with the nucleotide sequences of the corresponding genes of strains 2603 V/R and NEM316. Additionally, many nucleotide mutations were detected in the *pbp1a* genes, especially those from strains R7 and R8. Among the penicillin-susceptible strains, only strain N3 carried many nucleotide mutations, and these were especially detected in the *pbp1a* gene. From the viewpoint of amino acid substitutions, many of the nucleotide mutations detected in *pbp2a* were silent (Table 3).

A phylogenic tree constructed with the entire *pbp* sequences showed that the *pbp2x* and *pbp2b* genes of three groups of strains (strains R1, R2, R5, and R6; strains R3 and R4; and strains R7 and R8) and penicillin-susceptible strains N1, N2, N4, C1, C2, and 2603 V/R formed distinct genetic lineages. In particular, the *pbp2b* as well as the *pbp1a* genes of strains R7 and R8 (MIC, 1 μg/ml) formed a lineage distinct from the major lineage that included the other strains. The *pbp2x*, *pbp2b*, *pbp1a*, and *pbp2a* genes of penicillin-susceptible strain N3 belonged to a distinct lineage (Fig. 1). The phylogeny of the *pbp1b* gene showed no notable relationship with penicillin susceptibility (data not shown).

Amino acid substitutions in PBP 2X, PBP 2B, and PBP 1A. Figure 2 shows the amino acid substitutions in PBP 2X, PBP 2B, PBP 1A, PBP 2A, and PBP 1B identified in comparison with the published sequences, as described in the Materials and Methods. Except for strains N1 and N3, no amino acid substitutions were found in PBP 2X, PBP 2B, or PBP 1A of the penicillin-susceptible strains, irrespective of the dates of their isolation except. Strains N1 and N3 possessed one and four substitutions, respectively. The amino acid substitutions found in strains N1 and N3 were not found among the strains with reduced penicillin susceptibility. Of the six penicillin-insusceptible strains for which the penicillin MICs were 0.25 to 0.5 μg/ml, strains R1, R2, R5, and R6 shared a unique set of five substitutions, F395L, V405A, R433H, H438Y, and G648A in PBP 2X and a T567I substitution in PBP 2B. The first four substitutions were located in the transpeptidase domain of PBP 2X, and among these, F395L and V405A were at positions close to the $_{402}$SSN$_{404}$ motif. Only strain R6 had an additional substitution, A374V, in PBP 2X. The remaining strains, strains R3 and R4, shared Q557E, which is in proximity to the $_{552}$KSG$_{554}$ motif of PBP 2X, in addition to the T567I substitution in PBP 2B, which was commonly found among the six penicillin-insusceptible strains. Other substitutions detected were A400V, close to the $_{402}$SSN$_{404}$ motif of PBP 2X, and G539E in PBP 2B for R3 and Y262N in PBP 2B for R4. Among those six strains, no amino acid substitutions were detected in PBP 1A. A total of eight substitutions were shared by two penicillin-insusceptible strains, strains R7 and R8, for which the penicillin MICs were 1 μg/ml. Among those substitutions, seven substitutions (T77I and S353F in PBP 2X; V80A and G613R in PBP 2B; and L45P, N163K, and N723S in PBP 1A) were unique to those strains. The S353F and G613R substitutions were in proximity to the $_{344}$STMK$_{347}$ motif of PBP 2X and the $_{614}$KTG$_{616}$ motif of PBP 2B, respectively. Another substitution in PBP 2X, Q557E, was common to strains R3 and R4. There were two additional substitutions in each of two strains: F395V in PBP 2X and Y470F in PBP 1A for strain R7 and A514V in PBP 2X and G527V in PBP 1A for strain R8.

Furthermore, the sequence data for PBP 1A revealed that five amino acid residues (residues 718 to 722) consisting of NGNGN in strain 2603 V/R differed among NEM316 and the other strains tested, regardless of their penicillin susceptibility status.

Amino acid substitutions in PBP 2A and PBP 1B. In PBP 2A, an E63K substitution was observed in four of eight clinical strains with reduced penicillin susceptibilities as well as in all penicillin-susceptible strains except strain N3, which had three other substitutions. One substitution each was found in strain R4 (Y236C) and strain R6 (L285F). Strains R7 and R8 shared the T175I substitution. Sequencing data for PBP 1B showed that an L41S substitution was shared by six of eight clinical strains with reduced penicillin susceptibilities and by all penicillin-susceptible strains except strain N3 (Fig. 2).

Detection of PBPs and immunodetection of PBP 2X. Figure 3A shows the PBP profiles detected by the Bocillin FL-binding

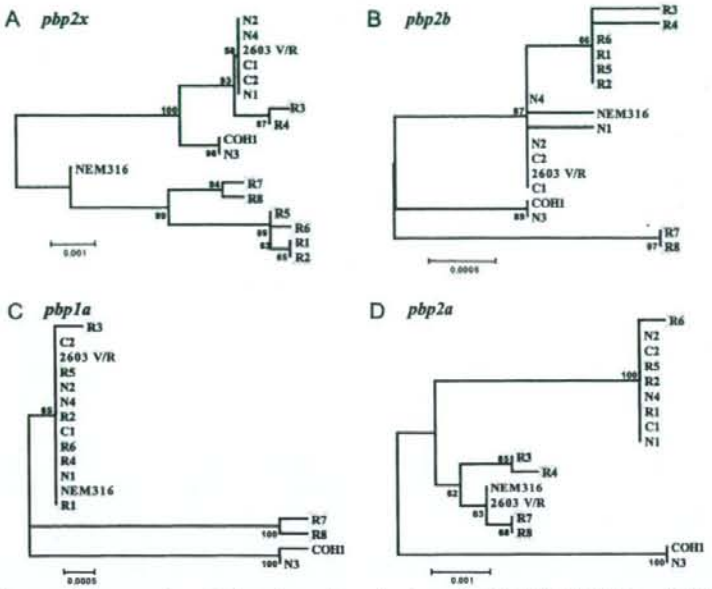

FIG. 1. Nucleotide phylogram of *pbp* genes from GBS strains and *S. agalactiae* strains 2603 V/R, NEM316, and COH1. Penicillin-insusceptible strains R1 to R8 are shaded. The nucleotide sequences were aligned by using the Clustal W program and were subjected to phylogenetic analysis by the neighbor-joining method based on Kimura's two-parameter model distance matrices with the MEGA program (version 3.1). The resulting trees were bootstrapped 500 times, and the bootstrap values are shown as percentages. The scale bars indicate the expected number of changes per sequence position.

assays. Five bands with molecular masses of 84, 77, 72, and 66 kDa (high-molecular-mass PBPs) and 42 kDa (low-molecular-mass PBP) were detected in strain 2603 V/R, in which strong signals were produced from the 77-kDa band, which comprised overlapping bands of two PBPs on 10% SDS-PAGE and which thus indicates that six bands were detected by the Bocillin FL-binding assays. The PBP profiles of the penicillin-insusceptible strains were similar to those of susceptible strains of 2603 V/R, C1, and C2, except for the lowering of the intensities of some bands among the high-molecular-mass PBPs for strains R1 to R8 in an analysis on a 5% SDS-PAGE gel, on which the highest-molecular-mass band consisted of two overlapping PBPs (Fig. 3B). Thus, the intensities of the second-highest-molecular-mass band were decreased 60% and 30% in strains

Strain	MIC (μg/ml)			PBP2X	PBP2B	PBP1A	PBP2A	PBP1B
	PEN	AMP	CTX	733344445556 6 / 757900331152 4 / 347505380477 8	812556 6 / 096361 2 / 022973 5	414456 7 / 565728718-22 2 / 533072 3	661223 3 / 397381 4 / 565666	49 / 15
2603 V/R	0.06	0.12	≤0.06	TSAIFAVRHVAQGG	VNYGTGV	LNSYGN NGNGN N	EPTYLKR	LA
NEM316	0.06	0.12	≤0.06	V I		NGN		D
N1	0.06	0.12	0.12		S	NGN	K	SD
N2	0.06	0.25	0.12			NGN	K	SD
N3	0.06	0.12	0.12	V V	I	N D N	L QK	
N4	0.06	0.12	≤0.06			NGN	K	SD
C1	0.06	0.12	≤0.06			NGNGN	K	SD
C2	0.06	0.25	0.12			NGN	K	SD
R1	0.5	0.5	0.5	VL AHYI A	I	NGN	K	SD
R2	0.5	0.5	0.5	VL AHYI A	I	NGN	K	SD
R3	0.5	0.25	0.5	V E	EI	NGN		SD
R4	0.5	0.25	0.5	E	NI	NGN	C	SD
R5	0.25	0.5	0.5	VL AHYI A	I	NGN	K	SD
R6	0.5	0.5	0.5	VVL AHYI A	I	NGN	K F	SD
R7	1	0.5	1	IF VV I E A	R	PK F N S	I	D
R8	1	0.5	1	IF V IVE A	R	PK V N S	I	D

FIG. 2. Deduced amino acid substitutions in five high-molecular-mass PBPs from GBS isolates. Residues mutated in PBPs from penicillin-insusceptible strains are shaded. Strain 2603 V/R is ATCC BAA-611, and strain NEM316 is ATCC 12403. PEN, penicillin; AMP, ampicillin; CTX, cefotaxime.

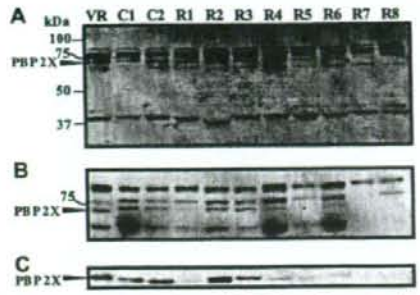

FIG. 3. Binding of penicillin to GBS PBPs and immunodetection of PBP 2X. Membrane proteins were incubated with Bocillin FL, separated on a 10% (A) or a 5% (B) SDS-polyacrylamide gel, and detected by fluorography, as described in Materials and Methods. The molecular sizes of the protein standards (Precision Plus; Bio-Rad Laboratories, Hercules, CA) are provided on the left. (C) Membrane proteins separated on a 5% SDS-polyacrylamide gel were subjected to Western blotting with anti-PBP 2X antibody as the primary antibody, as described in Materials and Methods. Arrows indicate the position of PBP 2X. VR, *S. agalactiae* 2603 V/R.

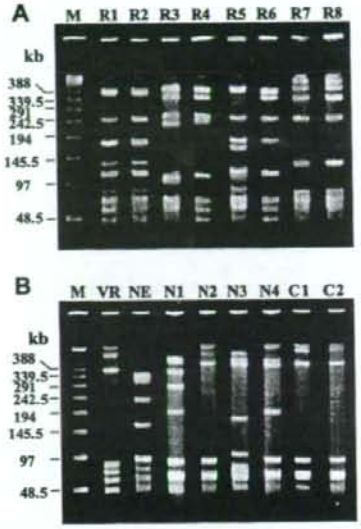

FIG. 4. PFGE profiles of genomic DNA of penicillin-insusceptible (A) and -susceptible (B) GBS isolates digested with SmaI. Lanes M, bacteriophage lambda DNA ladder as molecular size markers; VR, *S. agalactiae* 2603 V/R; NE, *S. agalactiae* NEM316.

R7 and R8, respectively. The intensity of the third band (molecular mass, 72 kDa) was decreased 30 to 60% in strains R1 to R6 or the band was not recognized in strains R7 and R8. The intensity of the fourth band (molecular mass, 66 kDa) was decreased 40 to 60% in strains R1 to R3 but was not recognized in strains R7 and R8. As shown in Fig. 3C, a positive reaction was obtained with the 72-kDa band by immunoblotting of the membrane proteins of strains probed with anti-PBP 2X antibodies recognizing a conserved 14-residue epitope. However, the amount of the antibody bound to the 72-kDa band was not equivalent among strains. Separately from the six PBPs, unstable Bocillin FL binding to a 45-kDa band was observed among all strains tested (data not shown).

***pbp2x* gene expression.** Expression of the *pbp2x* gene was quantitatively assessed by real-time RT-PCR. The relative amounts of *pbp2x* transcripts in penicillin-susceptible strains 2603 V/R, C1, and C2 ranged from 0.79 to 1.00, with a mean ± standard deviation of 0.88 ± 0.11, while those in penicillin-insusceptible strains R1 to R8 ranged from 0.74 to 1.32, with a mean ± standard deviation of 1.03 ± 0.23. No statistically significant differences in relative transcript levels were detected between these two groups (data not shown).

PFGE analysis. Analysis of SmaI-digested genomic DNA revealed six distinguishable patterns among all eight penicillin-insusceptible strains (Fig. 4A). Strains R1 and R2, which were serotype VI and which were detected from cultures of transtracheal aspirates from different inpatients in the neurosurgery ward of the Funabashi Municipal Medical Center over a 1-month interval, shared a pulsed-field gel electrophoresis (PFGE) profile. Strain R7, which was of serotype Ia, and nontypeable strain R8 shared another PFGE profile. Of note, these two strains originated from different inpatients in the same hospital (hospital D) 8 months apart. These findings were in agreement with the ApaI profiles (data not shown). Penicillin-susceptible strains did not share any PFGE profiles with penicillin-insusceptible strains (Fig. 4B). The PFGE profiles of strains C1 and C2, which had different serotypes, were largely similar by PFGE with SmaI but differed by more than 10 distinguishable bands by PFGE with ApaI (data not shown).

Epidemiology. The distributions of the MICs of representative β-lactams for 442 recent clinical isolates are shown in Table 4. There was a unimodal distribution for each β-lactam, with an elevated MIC outside the specified susceptible range (4, 5). Reduced susceptibilities to penicillin (MICs, >0.12 μg/ml), ampicillin (MICs, >0.25 μg/ml), and cefepime (MICs, >0.5 μg/ml) were demonstrated for 2.3%, 2.3%, and 0.9% of the isolates, respectively.

DISCUSSION

High rates of tetracycline resistance are noted in GBS, and increasing incidences of macrolide resistance as well as the emergence of fluoroquinolone resistance have been reported in recent years (3, 9, 14, 18, 24, 40). Although this study did not focus on quinolone-resistant strains, a trend toward increasing fluoroquinolone MICs has clearly been noted among recently emerged strains. However, confirmed resistance to β-lactams, including penicillin, has not been recognized among GBS isolates, although strains with reduced susceptibilities have been described in several reports (7, 16, 22). This is exemplified by the fact that the CLSI lists interpretive criteria for susceptibility for strains of beta-hemolytic streptococci, that is, MICs of ≤0.12 μg/ml, ≤0.25 μg/ml, and ≤0.5 μg/ml, only for penicillin, ampicillin, and cephems (cefotaxime, cefepime, and ceftriaxone) and meropenem, respectively; and it comments that strains with MICs greater than those breakpoints have not been observed (5). Very recently, Kimura et al., on the basis of molecular-based analyses, first described the alterations in high-molecular-mass PBPs in GBS clinical isolates exhibiting

TABLE 4. Distribution of MICs of β-lactams against GBS clinical isolates[a]

Antimicrobial agent	No. (%) of strains with MIC (μg/ml) of:						
	≤0.03	0.06	0.12	0.25	0.5	1	2
Penicillin	34 (7.7)	280 (63.3)	118 (26.7)	7 (1.6)	3 (0.7)		
Ampicillin		52[b] (11.8)	313 (70.8)	67 (15.1)	10 (2.3)		
Cefotaxime		301[b] (68.1)	114 (25.8)	16 (3.6)	11 (2.5)		
Cefepime					438[b] (99.1)	3 (0.7)	1 (0.2)
Cefozopran		35[b] (7.9)	316 (71.5)	72 (16.3)	17 (3.8)	2 (0.5)	
Cefditoren		399[b] (90.3)	25 (5.7)	11 (2.5)	6 (1.3)	1 (0.2)	
Meropenem[c]			365[b] (94.6)	19 (4.9)	2 (0.5)		

[a] A total of 442 isolates were tested.
[b] Includes isolates with MICs less than the given value.
[c] The number of isolates tested was 386.

increased MICs of β-lactams and the major role of altered PBP 2X in reducing their susceptibilities to these agents (19). In the present study, we investigated the amino acid sequences of five high-molecular-mass PBPs among clinical isolates with reduced susceptibilities to penicillin recovered during 2003 and 2004 in five geographically separate hospitals. The analysis included penicillin-susceptible strains comprising four strains from neonates with systemic infections isolated from 1976 to 1988 and two recently isolated strains. Thus, this study allowed us to verify whether or not the amino acid substitutions in PBPs reported previously (19) were common to our penicillin-insusceptible isolates and to investigate whether or not alterations in PBPs have accumulated over time, as well as whether such alterations could mediate the insusceptibilities to penicillins.

The deduced amino acid sequences of PBP 2X, PBP 2B, PBP 1A, and PBP 2A were highly conserved among six penicillin-susceptible strains, although strains N1 and N3 possessed one and seven substitutions, respectively. Because these amino acid substitutions were not found among the penicillin-insusceptible strains tested, they are probably unrelated to penicillin resistance. Of the six penicillin-insusceptible strains for which the penicillin MICs were 0.25 to 0.5 μg/ml, strains R1, R2, R5, and R6 shared a unique set of five amino acid substitutions, including V405A in PBP 2X and one in PBP 2B, that have been identified in some GBS isolates with reduced penicillin susceptibilities (19). The remaining two strains, strains R3 and R4, shared the Q557E substitution in PBP 2X, in addition to the T567I substitution in PBP 2B, which was commonly found among the six penicillin-insusceptible strains. Strains R7 and R8, for which the penicillin MICs were 1 μg/ml, shared a unique set of eight amino acid substitutions (two each in PBP 2X and PBP 2B, three in PBP 1A, and one in PBP 2A), in addition to the Q557E substitution in PBP 2X that was common to strains R3 and R4. Thus, our study confirmed previous findings about PBP alterations (19), and some other amino acid substitutions were also suggested to be involved in the elevation in the rate of penicillin insusceptibility. Our findings did not include amino acid substitutions within conserved motifs. However, the Q557E substitution, which was in proximity to the $_{552}$KSG$_{554}$ motif of PBP 2X, corresponds to the Q552E substitution in *S. pneumoniae* that has been reported to be responsible for the most of the reduction in susceptibility to β-lactams (28). The significance of each amino acid substitution located adjacent to conserved motifs, such as S353F, F395L, and A400V in PBP 2X and G613R in PBP 2B, needs to be assessed in future studies. Moreover, it will be necessary to look into additional possibilities that other substitutions located in the transpeptidase domain might also contribute to penicillin insusceptibility by providing compensatory structural and functional alterations in PBPs.

The amino acid substitutions found in PBPs imply that during evolution three groups of strains (strains R1, R2, R5, and R6; strains R3 and R4; and strains R7 and R8) each accumulated different genetic mutations for the acquisition of penicillin insusceptibility, and this finding is also suggested by the phylogram. Strains R1 and R2 had indistinguishable PFGE profiles as well as completely identical nucleotide sequences in their *pbp* genes; hence, their nosocomial transmission was strongly suspected. Strains R7 and R8 were isolated from different patients in the same hospital over an 8-month interval and shared the same PFGE profile. However, these two strains each had one different substitution in PBP 2X and PBP 1A and also shared many substitutions, which may lead us to speculate that they are derivatives of a strain that independently accumulated mutations, which allowed them to have common substitutions. These penicillin-insusceptible strains may possibly survive stably and could be further transmitted among patients in a clinical environment. The nucleotide sequences of the PBP 2X, PBP 2B, and PBP 1A genes of five of the six penicillin-susceptible strains, the exception being strain N3, were highly homologous with those of two penicillin-susceptible reference strains, irrespective of the dates of their isolation, and mostly fell into the same phylogenetic groups. In contrast, strain N3, which carried altered *pbp2x*, *pbp2b*, *pbp1a*, and *pbp2a* genes, formed an independent evolutionary lineage in the phylogram. In previous studies, it was demonstrated that this strain fell into a certain genotype among serotype III GBS strains which had been found at a high frequency in severe neonatal infections and was suggested to be a more virulent genotype than the other strains included in this serotype (35, 36). Interestingly, the nucleotide sequences of the *pbp* genes of strains N3 were almost 100% identical to the homologous genes of *Sreptococcus agalactiae* COH1 (GenBank accession number AAJR01000000), a highly virulent serotype III wild-type strain recovered from a neonatal patient with sepsis. Thus, they formed a distinct genetic lineage (Fig. 1). Few studies have addressed the role of PBPs in the pathogenicity of GBS. Jones et al. found that the *pon*A gene, which encodes extracytoplasmic PBP 1A, promoted resistance to phagocytic killing inde-

pendently of the capsular polysaccharide (17). Our results are of interest from the standpoint of the analysis of a possible association between PBPs and pathogenicity in this organism.

Six Bocillin FL-labeled PBPs with estimated molecular masses of 84, 77 (which comprised overlapping bands of two PBPs), 72, 66, and 42 kDa on a 10% SDS-polyacrylamide gel were detected in strain 2603 V/R as well as in penicillin-susceptible strains, which is somewhat similar to PBP patterns with molecular masses of 86 (which comprised overlapping bands of two PBPs), 80, 76, 70, and 42 kDa identified in a GBS strain with ^{3}H-labeled penicillin (15), while four PBPs with molecular masses of 101, 90, 82, and 66 kDa have been visualized in a GBS strains by Bocillin FL labeling (17). Among the penicillin-insusceptible strains, the decrease or the absence of intensities for some bands was noted among high-molecular-mass PBPs. A correlation was found between the decrease in the levels of the band intensities and the increase in the penicillin MICs, so the most likely explanation for the phenomenon is that altered PBPs may reduce their affinities for Bocillin FL. However, unexpectedly, the amount of the anti-PBP 2X antibody bound to the 72-kDa PBP was not equivalent among strains but, rather, paralleled the band intensity of Bocillin FL-labeled PBP 2X. If the amount of antibody bound to PBP 2X reflects the amount of PBP 2X, then the possibility that an altered PBP 2X or that the intrinsic instability of an altered PBP 2X results in a reduction in the levels of expression cannot be excluded. However, the relative levels of the *pbp2x* transcript between penicillin-insusceptible and -susceptible strains were statistically indistinguishable by Student's *t* test analysis, indicating that such a decrease in the signal intensity of Bocillin FL for altered PBP 2X cannot be accounted for by a reduction in the level of expression. Complete understanding would require examination of whether or not the stability is affected in the altered PBP 2X, then this study should be expanded to PBP 2B, PBP 1A, and PBP 2A, although no antibodies for these molecules are presently available.

Neonatal GBS meningitis is a life-threatening infection, and even if the treatment is started in the early stage, it has a poor prognosis, in that many cases may develop severe neurological sequelae. Rapid and accurate clinical diagnosis is therefore crucial, and most clinical laboratory efforts have focused on the prompt identification of the causative agent by performing a rapid antigen detection test with cerebrospinal fluid specimens as well as Gram staining. Once the test result is positive for the GBS antigen, specific therapy is initiated with penicillin or ampicillin plus gentamicin (30, 38), because GBS is considered to be uniformly susceptible to these drugs. In the present study, no penicillin insusceptibility was detected among invasive GBS isolates. In addition, the clinical significance of the in vitro penicillin insusceptibility of invasive isolates in antimicrobial chemotherapy and also in intrapartum prophylaxis remains unclear. However, our epidemiological findings revealed a unimodal MIC distribution for β-lactams, including penicillin, with the MIC elevated above the specified susceptible range. Hence, the emergence of such strains with reduced penicillin susceptibility should trigger awareness of the appropriate therapeutic strategy for dealing with severe GBS infections and the strategy for intrapartum prophylaxis for GBS carriers.

ACKNOWLEDGMENTS

This work was supported by a grant (H18-Shinkou-011) from the Ministry of Health, Labor and Welfare of Japan.

We are grateful to Kunikazu Yamane, Jun-ichi Wachino, and Naohiro Shibata for technical advice.

REFERENCES

1. **Appelbaum, P. C.** 1992. Antimicrobial resistance in Streptococcus pneumoniae: an overview. Clin. Infect. Dis. **15**:77–83.
2. **Appelbaum, P. C., A. Bhamjee, J. N. Scragg, A. F. Hallett, A. J. Bowen, and R. C. Cooper.** 1977. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. Lancet **ii**:995–997.
3. **Biedenbach, D. J., J. M. Stephen, and R. N. Jones.** 2003. Antimicrobial susceptibility profile among beta-haemolytic Streptococcus spp. collected in the SENTRY Antimicrobial Surveillance Program—North America, 2001. Diagn. Microbiol. Infect. Dis. **46**:291–294.
4. **Clinical and Laboratory Standards Institute.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6, 6th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2007. Performance standards for antimicrobial susceptibility testing. Supplement M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Dahesh, S., M. E. Hensler, N. M. Van Sorge, R. E. Gertz, Jr., S. Schrag, V. Nizet, and B. W. Beall.** 2008. Point mutation in the group B streptococcal *pbp2x* gene conferring decreased susceptibility to β-lactam antibiotics. Antimicrob. Agents Chemother. **52**:2915–2918.
7. **De Azavedo, J. C., M. McGavin, C. Duncan, D. E. Low, and A. McGeer.** 2001. Prevalence and mechanisms of macrolide resistance in invasive and noninvasive group B streptococcus isolates from Ontario, Canada. Antimicrob. Agents Chemother. **45**:3504–3508.
8. **Farley, M. M., R. C. Harvey, T. Stull, J. D. Smith, A. Schuchat, J. D. Wenger, and D. S. Stephens.** 1993. A population-based assessment of invasive disease due to group B *Streptococcus* in nonpregnant adults. N. Engl. J. Med. **328**: 1807–1811.
9. **Fernandez, M., M. E. Hickman, and C. J. Baker.** 1998. Antimicrobial susceptibilities of group B streptococci isolated between 1992 and 1996 from patients with bacteremia or meningitis. Antimicrob. Agents Chemother. **42**:1517–1519.
10. **Goffin, C., and J. M. Ghuysen.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62**:1079–1093.
11. **Graham, M. R., L. M. Smoot, C. A. Migliaccio, K. Virtaneva, D. E. Sturdevant, S. F. Porcella, M. J. Federle, G. J. Adams, J. R. Scott, and J. M. Musser.** 2002. Virulence control in group A streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. Proc. Natl. Acad. Sci. USA **99**:13855–13860.
12. **Grebe, T., and R. Hakenbeck.** 1996. Penicillin-binding proteins 2B and 2X of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob. Agents Chemother. **40**:829–834.
13. **Hansman, D., and M. M. Bullen.** 1967. A resistant pneumococcus. Lancet **ii**:264–265.
14. **Heelan, J. S., M. E. Hasenbein, and A. J. McAdam.** 2004. Resistance of group B streptococcus to selected antibiotics, including erythromycin and clindamycin. J. Clin. Microbiol. **42**:1263–1264.
15. **Horne, D., and A. Tomasz.** 1981. pH-dependent penicillin tolerance of group B streptococci. Antimicrob. Agents Chemother. **20**:128–135.
16. **Hsueh, P.-R., L.-J. Teng, L.-N. Lee, S.-W. Ho, P.-C. Yang, and K.-T. Luh.** 2001. High incidence of erythromycin resistance among clinical isolates of *Streptococcus agalactiae* in Taiwan. Antimicrob. Agents Chemother. **45**: 3205–3208.
17. **Jones, A. L., R. H. Needham, A. Clancy, K. M. Knol, and C. E. Rubens.** 2003. Penicillin-binding proteins in *Streptococcus agalactiae*: a novel mechanism for evasion of immune clearance. Mol. Microbiol. **47**:247–256.
18. **Kawamura, Y., H. Fujiwara, N. Mishima, Y. Tanaka, A. Tanimoto, S. Ikawa, Y. Itoh, and T. Ezaki.** 2003. First *Streptococcus agalactiae* isolates highly resistant to quinolones with point mutations in *gyrA* and *parC*. Antimicrob. Agents Chemother. **47**:3605–3609.
19. **Kimura, K., S. Suzuki, J. Wachino, H. Kurokawa, K. Yamane, N. Shibata, N. Nagano, H. Kato, K. Shibayama, and Y. Arakawa.** 2008. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob. Agents Chemother. **52**:2890–2897.
20. **Klugman, K. P.** 1990. Pneumococcal resistance to antibiotics. Clin. Microbiol. Rev. **3**:171–196.
21. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5**:150–163.
22. **Morikawa, Y., M. Kitazato, C. Katsukawa, and A. Tamaru.** 2003. Prevalence of cefotaxime resistance in group B streptococcus isolates from Osaka, Japan. J. Infect. Chemother. **9**:131–133.

23. **Munoz, R., C. G. Dowson, M. Daniels, T. J. Coffey, C. Martin, R. Hakenbeck, and B. G. Spratt.** 1992. Genetics of resistance to third-generation cephalosporins in clinical isolates of *Streptococcus pneumoniae*. Mol. Microbiol. **6**:2461–2465.
24. **Murdoch, D. R., and L. B. Reller.** 2001. Antimicrobial susceptibilities of group B streptococci isolated from patients with invasive disease: 10 year perspective. Antimicrob. Agents Chemother. **45**:3623–3624.
25. **Nagano, N., N. Shibata, Y. Saitou, Y. Nagano, and Y. Arakawa.** 2003. Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type β-lactamase. J. Clin. Microbiol. **41**:5530–5536.
26. **Nagano, N., Y. Nagano, and F. Taguchi.** 2002. High expression of a C protein β antigen gene among invasive strains from certain clonally related groups of type Ia and Ib group B streptococci. Infect. Immun. **70**:4643–4649.
27. **Nagano, N., Y. Nagano, R. Nakano, R. Okamoto, and M. Inoue.** 2006. Genetic diversity of the C protein β-antigen gene and its upstream regions within clonally related groups of type Ia and Ib group B streptococci. Microbiology **152**:771–778.
28. **Pernot, L., L. Chesnel, A. Le Gouellec, J. Croize, T. Vernet, O. Dideberg, and A. Dessen.** 2004. A PBP2x from a clinical isolate of *Streptococcus pneumoniae* exhibits an alternative mechanism for reduction of susceptibility to beta-lactam antibiotics. J. Biol. Chem. **279**:16463–16470.
29. **Regan, J. A., M. A. Klebanoff, R. P. Nugent, et al.** 1991. The epidemiology of group B streptococcal colonization in pregnancy. Obstet. Gynecol. **77**:604–610.
30. **Saez-Llorens, X., and G. H. McCracken, Jr.** 2003. Bacterial meningitis in children. Lancet **361**:2139–2148.
31. **Schuchat, A.** 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin. Microbiol. Rev. **11**:497–513.
32. **Schuchat, A.** 1999. Group B streptococcus. Lancet **353**:51–56.
33. **Smith, A. M., and K. P. Klugman.** 1998. Alterations in PBP 1A essential for high-level penicillin resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **42**:1329–1333.
34. **Spratt, B. G., and K. D. Cromie.** 1988. Penicillin-binding proteins of gram-negative bacteria. Rev. Infect. Dis. **10**:699–711.
35. **Takahashi, S., E. E. Adderson, Y. Nagano, N. Nagano, M. R. Briesacher, and J. F. Bohnsack.** 1998. Identification of a highly encapsulated, genetically related group of invasive type III group B streptococci. J. Infect. Dis. **177**: 1116–1119.
36. **Takahashi, S., Y. Nagano, N. Nagano, O. Hayashi, F. Taguchi, and Y. Okuwaki.** 1995. Role of C5a-ase in group B streptococcal resistance to opsonophagocytic killing. Infect. Immun. **63**:4764–4769.
37. **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.
38. **Tunkel, A. R., B. J. Hartman, S. L. Kaplan, B. A. Kaufman, K. L. Roos, W. M. Scheld, and R. J. Whitley.** 2004. Practice guidelines for the management of bacterial meningitis. Clin. Infect. Dis. **39**:1267–1284.
39. **van Asselt, G. J., G. de Kort, and J. A. M. van de Klundert.** 1995. Differences in penicillin-binding protein patterns of penicillin tolerant and nontolerant group A streptococci. J. Antimicrob. Chemother. **35**:67–74.
40. **Wehbeh, W., R. Rojas-Diaz, X. Li, N. Mariano, L. Grenner, S. Segal-Maurer, B. Tommasulo, K. Drlica, C. Urban, and J. J. Rahal.** 2005. Fluoroquinolone-resistant *Streptococcus agalactiae*: epidemiology and mechanism of resistance. Antimicrob. Agents Chemother. **49**:2495–2497.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2009, p. 69–74
0066-4804/09/$08.00+0 doi:10.1128/AAC.00227-08

Vol. 53, No. 1

Novel Chimeric β-Lactamase CTX-M-64, a Hybrid of CTX-M-15-Like and CTX-M-14 β-Lactamases, Found in a *Shigella sonnei* Strain Resistant to Various Oxyimino-Cephalosporins, Including Ceftazidime[▽]

Yukiko Nagano,[1] Noriyuki Nagano,[1,2] Jun-ichi Wachino,[1] Keiko Ishikawa,[3] and Yoshichika Arakawa[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] Medical Microbiology Laboratory, Funabashi Municipal Medical Center, Funabashi, Chiba,[2] and Clinical Microbiology Laboratory, Urayasu Ichikawa City Hospital, Urayasu, Chiba,[3] Japan

Received 19 February 2008/Returned for modification 24 March 2008/Accepted 18 October 2008

The plasmid-mediated novel β-lactamase CTX-M-64 was first identified in *Shigella sonnei* strain UIH-1, which exhibited resistance to cefotaxime (MIC, 1,024 μg/ml) and ceftazidime (MIC, 32 μg/ml). The amino acid sequence of CTX-M-64 showed a chimeric structure of a CTX-M-15-like β-lactamase (N- and C-terminal moieties) and a CTX-M-14-like β-lactamase (central portion, amino acids 63 to 226), suggesting that it originated by homologous recombination between the corresponding genes. The introduction of a recombinant plasmid carrying $bla_{CTX-M-64}$ conferred resistance to cefotaxime in *Escherichia coli*, and the activities of cefotaxime and ceftazidime were restored in the presence of clavulanic acid. Of note, CTX-M-64 production could also confer consistent resistance to ceftazidime, which differs from the majority of CTX-M-type enzymes, which poorly hydrolyze ceftazidime. These results were consistent with the kinetic parameters determined with the purified CTX-M-64 enzyme. The $bla_{CTX-M-64}$ gene was flanked upstream by an IS*Ecp1* sequence and downstream by an *orf477* sequence. The sequence of the 45-bp spacer region between the right inverted repeat (IRR) of IS*Ecp1* and $bla_{CTX-M-64}$ was exactly identical to that of IS*Ecp1*-$bla_{CTX-M-15-like}$. Moreover, the presence of a putative IRR of IS*Ecp1* at the right end of truncated *orf477* is indicative of an IS*Ecp1*-mediated transposition event in the $bla_{CTX-M-64}$ gene. The emergence of CTX-M-64 by probable homologous recombination would suggest the natural potential of an alternative mechanism for the diversification of CTX-M-type β-lactamases.

Shigellosis remains a public health concern throughout the world and has become an actual threat, particularly in developing countries, where 99% of the estimated 165 million annual episodes occur. Children under 5 years of age have been involved in more than half of the episodes and deaths (14). Shigellosis is more severe in malnourished children and elderly and immunocompromised people. Antibiotic treatment shortens the duration of clinical symptoms and pathogen excretion, prevents disease transmission, and reduces the risk of potential complications (18, 27, 34). However, empirical therapy with first-line antimicrobial agents, including ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, nalidixic acid, co-trimoxazole, and tetracycline, has become less effective due to the high prevalence of multidrug-resistant (MDR) clinical isolates among *Shigella* species (9, 28, 29, 32). For these MDR isolates, the therapeutic options for oral administration are fluoroquinolones for adults and oxyimino-cephalosporins for children.

Plasmid-encoded class A extended-spectrum β-lactamase (ESBL) production is still uncommon among *Shigella* species, despite the worldwide spread and prevalence of ESBL-producing clinical isolates belonging to the family *Enterobacteriaceae*. Four CTX-M-type β-lactamases, CTX-M-2, CTX-M-3, CTX-M-14, and CTX-M-15, and several TEM-derived ESBLs have been reported for *Shigella sonnei* (1, 11, 15, 25). *S. sonnei* strain UIH-1, characterized in this study, produced a novel CTX-M-type β-lactamase, a hybrid of the CTX-M-15-like β-lactamase, which is a new CTX-M-15 variant (GenBank accession no. DQ256091), and the CTX-M-14 β-lactamase; and this chimeric enzyme conferred resistance to ceftazidime as well as to cefotaxime and ceftriaxone.

MATERIALS AND METHODS

Clinical isolate. *S. sonnei* UIH-1 was identified with the API 20E system (bioMérieux) in combination with tests for the utilization of citrate with Christensen's citrate medium (4), sodium acetate, and mucate and by PCR detection of the *invE* and *ipaH* genes with specific primer sets (Takara Bio, Shiga, Japan). Serological identification was performed with specific antisera (Denka Seiken, Tokyo, Japan).

Susceptibility testing. β-Lactam MICs were measured by the microdilution broth method with a WalkAway-96 SI system (NEG Combo 6.11 J, NEG MIC 5 J, and ESBL plus panels; Dade Behring, Tokyo, Japan), according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (5, 21). Alternatively, for cefotaxime, ceftazidime, ceftriaxone, and aztreonam (Sigma), broth microdilution panels prepared in-house were used to provide a broader range of antimicrobial concentrations for evaluation of the MICs (5). Susceptibilities to non-β-lactams were tested by the disk diffusion method recommended by the CLSI (5). The susceptibility categories of the parent strain, the transformant, and the transconjugant were determined according to the criteria of the CLSI (6).

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 3500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

[▽] Published ahead of print on 27 October 2008.

PCR detection and sequencing of β-lactamase gene. Detection of the *bla* genes, including bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, and $bla_{CTX\text{-}M\text{-}8/25}$, was performed by PCR, as described previously (20). The additional primers used were consensus primers CTX-M/F' and CTX-M/R1 (22) and primers CTX-M1A and CTX-M1B, which encompass the entire coding region (8). For sequence determination, the amplicons were purified with a QIAquick PCR purification kit (Qiagen), and both strands were directly sequenced with a BigDye Terminator cycle sequencing ready reaction kit and an ABI Prism model 3100 genetic analyzer (Applied Biosystems). The nucleotide and deduced amino acid sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The ClustalW program (http://www.ebi.ac.uk/clustalw) was used to align the amino acid sequences of multiple enzymes.

Plasmid conjugal transfer. The conjugal transferability of the resistance determinants was investigated as described previously (20). Transconjugants were selected on bromothymol blue-lactose agar containing cefotaxime (20 μg/ml) and rifampin (rifampicin; 100 μg/ml; Sigma).

Cloning of $bla_{CTX\text{-}M\text{-}64}$. The conjugal plasmid was extracted and digested with EcoRI. The resultant fragments were ligated into the pCL1920 cloning vector (GenBank accession no. AB236930) and introduced into *Escherichia coli* XL-1 Blue. The transformants were selected on LB agar plates containing streptomycin (25 μg/ml; Sigma) and ampicillin (100 μg/ml; Sigma). The $bla_{CTX\text{-}M\text{-}64}$ gene and its flanking region were amplified with the primers 5'-GGG GAT CCT TGC TCT GTG GAT AAC TTG CAG-3' (the KpnI site is underlined) and 5'-CCC AAG CTT TCG GTG CAT AAA ACA CGG TG-3' (the HindIII site is underlined). The product was digested with restriction enzymes and cloned into plasmid pCL1920. The resultant recombinant plasmid was introduced into *E. coli* XL-1 Blue, and transformants were selected as described above. To ensure that the enzyme was produced in the transformant, the nucleotide sequence of the insert was checked as described above.

Southern hybridization. Plasmid DNA was prepared from bacterial cells by the alkaline extraction method (20). The DNAs were transferred to a positively charged nylon membrane (Clearblot N$^+$ membrane; Atto Corp., Tokyo, Japan). The PCR product obtained with primers CTX-M/F' and CTX-M/R1 (22) was labeled with digoxigenin-11-dUTP by use of a DIG High Prime DNA labeling and detection kit (Roche Applied Science). Hybridization and detection were performed according to the manufacturer's recommendations.

Purification of CTX-M-64 β-lactamase. The $bla_{CTX\text{-}M\text{-}64}$ gene was amplified with primers P1 (5'-GGA ATT CCA TAT GGT TAA AAA ATC ACT GCG-3'), which introduced an NdeI restriction site (underlined) to the 5' end, and P2 (5'-CCC AAG CTT TTA CAA ACC GTC GGT GAC GAT-3') which introduced an HindIII site (underlined) to the 3' end. The amplified fragments were digested with the restriction enzymes and ligated into the pET29a vector (Novagen). Recombinant plasmid pET-CTX-M-64 was electroporated into *E. coli* BL21(DE3)pLysS after confirmation that the plasmid contained the $bla_{CTX\text{-}M\text{-}64}$ gene sequence by sequencing analysis. The cells were cultured in 1 liter of LB broth supplemented with chloramphenicol (30 μg/ml; Sigma) and kanamycin (30 μg/ml; Sigma) at 37°C. Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.5 mM) was added when the optical density of the culture at 600 nm reached 0.5, and the culture was incubated for an additional 3 h at 37°C. The cells were disrupted with a French press and centrifuged at 100,000 × *g* for 1 h. The supernatant containing the recombinant protein was loaded onto a HiTrap SP HP column (GE Healthcare) that had been preequilibrated with 50 mM morpholineethanesulfonic acid buffer (pH 6.0). The enzymes were eluted with a linear gradient of NaCl in the same buffer. The fractions with β-lactamase activity were loaded onto a Superdex 200 10/300GL column (GE Healthcare) and eluted with buffer (20 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1 mM dithiothreitol). Finally, the eluted protein was concentrated and stored at −80°C until use. The purity of the β-lactamase was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. The purified β-lactamase was also subjected to isoelectric focusing analysis with an Ampholine PAG plate (Amersham Biosciences).

Determination of kinetic parameters. The kinetic parameters for the CTX-M-64 β-lactamase against various β-lactam substrates were measured at 37°C in 50 mM phosphate buffer (pH 7.0) with a spectrophotometer (Ultrospec 3000; Pharmacia Biotech). The values of the kinetic parameters K_m and k_{cat} were obtained from a Michaelis-Menten plot of the initial steady-state velocities (7, 30). Six different substrate concentrations were used to determine the parameters for each substrate. The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; nitrocefin, 485 nm; cephalothin, 262 nm; ceftazidime, 274 nm; cefotaxime, 264 nm; and cefepime, 275 nm. The K_m of poor substrates was determined as the competitive inhibition constant (K_i) from the competition assay between the substrate (ceftazidime) and nitrocefin (100 μM). The 50% inhibitory concentration was determined as the concentration of clavulanic acid that reduced the hydrolysis rate of 100 μM nitrocefin by 50% when the enzyme was preincubated with various concentrations of the inhibitor for 5 min at 37°C before addition of the substrate.

Nucleotide sequence accession number. The nucleotide sequence data for $bla_{CTX\text{-}M\text{-}64}$ of *S. sonnei* UIH-1 appear in the DDBJ/EMBL/GenBank database under accession no. AB284167.

RESULTS

Description of clinical isolate. *S. sonnei* UIH-1 was recovered in August 2006 at the Urayasu Ichikawa City Hospital, Chiba, Japan, from a culture of a stool sample from a 37-year-old man with diarrhea, tenesmus, and fever that continued for 3 days after a trip to China. Serotyping revealed mostly smooth phase I colonies, but a small number of rough phase II variants were intermingled in the initial isolation culture. Both phase I and phase II strains of *S. sonnei* UIH-1 showed the same antibiograms with all antimicrobials tested. They were resistant to penicillins, cefotaxime (MIC, 1,024 μg/ml), ceftazidime (MIC, 32 μg/ml), ceftriaxone (1,024 μg/ml), cefpodoxime (MIC, >64 μg/ml), and aztreonam (32 μg/ml); and the activities of cefotaxime and ceftazidime were restored by clavulanic acid. The MICs of cephamycins, oxacephems, and carbapenems were within the susceptible range (Table 1). These isolates were also resistant to streptomycin, nalidixic acid, trimethoprim-sulfamethoxazole, and tetracycline.

PCR and sequencing of *bla* gene. A preliminary search for *bla* genes by the conventional PCR method failed to give positive results. However, PCR with $bla_{CTX\text{-}M}$-specific consensus primers allowed the detection of a 520-bp fragment. Except for the primer sequences, the 478-bp nucleotide sequence contained a 450-bp sequence from its 5' end that was identical to nucleotides 228 to 677 of the $bla_{CTX\text{-}M\text{-}9\ group}$ and a 31-bp sequence from its 3' end that was identical to nucleotides 675 to 705 of the $bla_{CTX\text{-}M\text{-}1\ group}$. On the basis of the finding that our $bla_{CTX\text{-}M}$ gene could have a $bla_{CTX\text{-}M\text{-}9\ group}$–$bla_{CTX\text{-}M\text{-}1\ group}$ hybrid sequence, all possible combinations of primers from our stock were used in an attempt to amplify the structural gene by PCR, which resulted in the generation of an amplicon of the expected size with primers CTX-M1A and CTX-M1B. The sequence data for $bla_{CTX\text{-}M}$ indicated the presence of an open reading frame of 876 bp encoding a protein consisting of 291 amino acid residues. A BLAST search revealed 100% identity with $bla_{CTX\text{-}M\text{-}15\text{-}like}$ (GenBank accession no. DQ256091) from nucleotides 1 to 209 and nucleotides 675 to 876 and 100% identity with $bla_{CTX\text{-}M\text{-}14}$ (GenBank accession no. AF252622) (or $bla_{CTX\text{-}M\text{-}17,\ -21,\ -24,\ -27}$) from nucleotides 202 to 677. The deduced amino acid sequence showed 100% identity to the CTX-M-15-like β-lactamase derived from CTX-M-15 (GenBank accession no. AY044436) through a single Ala67-Pro substitution from amino acid residues 1 to 82 and amino acid residues 223 to 290 and 100% identity to the CTX-M-14 β-lactamase (or the CTX-M-9, -16, -17, -21, -24, and -27 β-lactamases) from amino acid residues 63 to 226 (Fig. 1). CTX-M-14 was expected to be the most probable variant forming the middle hybrid part because the highest prevalence of this enzyme among the CTX-M-9 group of enzymes described above has been noted in the Far East (3, 16, 19, 35). Thus, this novel CTX-M-type β-lactamase has been assigned the designation CTX-M-64 by G. A. Jacoby (http://www.lahey.org/studies/webt.asp), which differed from the CTX-M-15-like β-lactamase by 22 amino acid residues (92.4% similarity) and

TABLE 1. MICs of β-lactams for *S. sonnei* clinical isolate UIH-1, the transconjugant, and the transformant

Antibiotic	MIC (μg/ml)				
	S. sonnei UIH-1	*E. coli* χ1037 Rif^r transconjugant	*E. coli* χ1037 Rif^r	*E. coli* XL-1 Blue(pCL1920-CTX-M-64)	*E. coli* XL-1 Blue(pCL1920)
Ampicillin	>16	>16	≤2	>16	≤2
Amoxicillin-CLA	2/1	2/1	≤1/0.5	8/4	2/1
Piperacillin	>64	>64	≤8	>64	≤8
Cefazolin	>16	>16	≤1	>16	≤1
Cefotiam	>16	>16	≤8	>16	≤8
Cefoperazone-SUL[a]	8/4	≤4/2	≤4/2	>32/16	≤4/2
Cefotaxime[c]	1,024	2,048	≤0.25	>2,048	≤0.25
Cefotaxime-CLA[b]	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Ceftazidime[c]	32	64	≤0.25	2,048	≤0.25
Ceftazidime-CLA[b]	≤0.12	≤0.12	≤0.12	≤0.5	≤0.12
Ceftriaxone[c]	1,024	2,048	≤0.25	2,048	≤0.25
Cefpirome	>16	>16	≤1	>16	≤1
Cefepime	>32	>32	≤1	>32	≤1
Cefozopran	>16	>16	≤1	>16	≤1
Cefaclor	>16	>16	≤2	>16	≤2
Cefpodoxime	>64	>64	≤0.5	>64	≤0.5
Cefoxitin	≤2	≤2	≤2	8	4
Cefmetazole	≤0.5	1	≤0.5	1	1
Cefotetan	≤0.5	≤0.5	≤0.5	8	≤0.5
Flomoxef	≤1	≤1	≤1	≤1	≤1
Imipenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Meropenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Aztreonam[c]	32	64	≤0.25	1,024	≤0.25

[a] SUL, sulbactam.
[b] CLA, clavulanic acid at a fixed concentration of 4 μg/ml.
[c] The antibiotic-containing plates were prepared in-house.

from the CTX-M-14 β-lactamase by 35 amino acid residues (88.0% similarity).

Genetic environment of $bla_{CTX\text{-}M\text{-}64}$. The flanking region of $bla_{CTX\text{-}M\text{-}64}$ was determined (Fig. 2). The sequence of the spacer region between the right inverted repeat (IRR) of IS*Ecp1* and $bla_{CTX\text{-}M\text{-}64}$ was exactly identical to that of IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15\text{-like}}$ (GenBank accession no. DQ256091), in which the length was 45 bp and in which there were 2 nucleotide substitutions from the corresponding IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$ spacer region of 48 bp (GenBank accession no. AY044436).

FIG. 1. Comparison of the amino acid sequences of the CTX-M-64 β-lactamase with those of the CTX-M-15-like and CTX-M-14 β-lactamases. The complete sequence of CTX-M-64 is shown, and only differences in the sequence are indicated for the other two enzymes. Structural elements characteristic of class A β-lactamases are boxed. The amino acids of the omega loop are underlined.

Cloning analysis revealed that a 345-bp 5′-truncated *orf477* was located 47 bp downstream of the $bla_{CTX\text{-}M\text{-}64}$ termination codon, in which the 5′ end of the *orf477* was terminated by an 18-bp putative IRR of IS*Ecp1* (5′-GCGCACGTAGGTCCCA GG-3′) that was identical to a previously described IRR (26). The 112-bp sequence located immediately downstream of the truncated *orf477* showed 100% identity with the sequence encoding the 3′ end of hypothetical protein 0115 and the start region of a hypothetical protein 0116 located on a large MDR plasmid of *Salmonella enterica* subsp. *enterica* serovar Newport (GenBank accession no. CP000604). The backbone plasmid of the MDR plasmid of 113,320 bp is shared by *Yersinia pestis* and has been detected in numerous MDR enterobacterial pathogens isolated from retail meat samples (31). The 112-bp sequence was followed by a 32-bp sequence which showed 90% identity with a 16-bp sequence encoding a 3′-truncated *yadD* homologue and a flanking spacer region of plasmid ColIb P-9 (GenBank accession no. AJ238399).

Transfer of cefotaxime resistance and plasmid DNA analysis. Cefotaxime resistance was transferred to *E. coli* at an approximate frequency of 1.1×10^{-5} CFU/donor cell. Electrophoretic analysis of the plasmid DNA revealed the transfer of a plasmid, and Southern blot hybridization analysis confirmed that the $bla_{CTX\text{-}M\text{-}64}$ gene was located on this approximately 68-kb plasmid (data not shown).

Susceptibility testing. The MICs of various β-lactams for the transconjugant and the transformant are listed in Table 1. Both the transconjugant and the transformant producing the CTX-M-64 enzyme conferred consistent resistance to cefotaxime, ceftriaxone, and aztreonam; on the other hand, they were susceptible to cephamycins and carbapenems. The reduction in

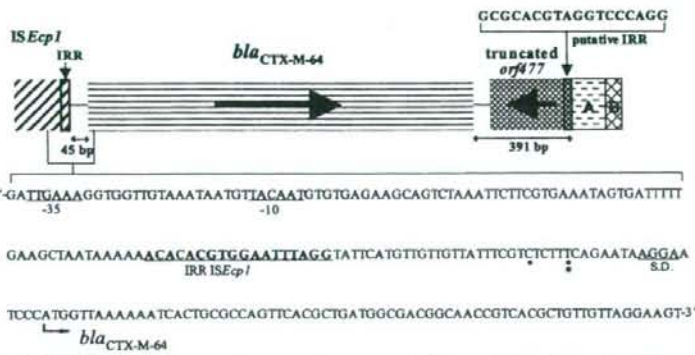

FIG. 2. Schematic diagram of the $bla_{CTX-M-64}$ gene and surrounding regions in *S. sonnei* UIH-1. For the upstream region of the $bla_{CTX-M-64}$ gene, the nucleotide sequence of the 3′ end of an IS*Ecp1* element and the start region of *bla* are indicated. Putative −35 and −10 promoter sequences of the $bla_{CTX-M-64}$ gene and the IRR sequence of IS*Ecp1* are underlined. A probable Shine-Dalgarno sequence (S.D.) is also underlined. Asterisks indicate the nucleotide substitutions from the corresponding IS*Ecp1*-$bla_{CTX-M-15}$ spacer sequence (GenBank accession no. AY044436). The closed circle indicates the nucleotide substitution from the corresponding chromosomal $bla_{CTX-M-3}$ spacer sequence of *Kluyvera ascorbata* (GenBank accession no. AJ632119). The 391-bp sequence of the downstream region of the *bla* gene is 100% identical to the corresponding sequences of the *K. ascorbata* chromosomal $bla_{CTX-M-3}$, including the putative IRR from IS*Ecp1*. The sequence is followed by a downstream region showing sequence homology with hypothetical proteins located on a large MDR plasmid of *Salmonella enterica* subsp. *enterica* serovar Newport (GenBank accession no. CP000604) (block A), a 3′-truncated *yadD* homologue, and the flanking spacer region of plasmid ColIb P-9 (GenBank accession no. AJ238399) (block B).

the MIC of cefotaxime was observed by the addition of clavulanic acid. This trend is commonly observed in the majority of CTX-M-type β-lactamase producers. Of note, the CTX-M-64-producing transformant had a considerably augmented MIC of ceftazidime, which is thought to be a poor substrate for most CTX-M-type β-lactamases.

Purification and characterization of CTX-M-64 β-lactamase. *E. coli* BL21(DE3)pLysS and the pET-29a vector were used for the overexpression of the $bla_{CTX-M-64}$ gene for the purification of CTX-M-64. The optimized culture conditions yielded approximately 7 mg of purified CTX-M-64 enzyme per liter. The purified CTX-M-64 gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the pI of the enzyme was determined to be >8.7 (data not shown).

Kinetic parameters. As shown in Table 2, CTX-M-64 showed high catalytic efficiencies (k_{cat}/K_m values) against ampicillin, nitrocefin, cephalothin, and cefotaxime, as is observed for other CTX-M-type β-lactamases. The 50% inhibitory concentration of clavulanic acid measured with nitrocefin as the substrate was 0.01 μM, and this result corroborated the inhibitor-sensitive nature of the CTX-M-64 enzyme. The catalytic activity (k_{cat}) of CTX-M-64 against ceftazidime could not be determined due to its very high K_i value.

TABLE 2. Kinetic parameter values for the CTX-M-64 β-lactamase

Substrate	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Ampicillin	19.5 ± 1.78	36.9 ± 0.89	1.9×10^6
Nitrocefin	14.8 ± 0.93	252 ± 5.10	1.7×10^7
Cephalothin	37.9 ± 1.06	185 ± 4.43	4.9×10^6
Cefotaxime	103 ± 13.6	197 ± 14.6	1.9×10^6
Ceftazidime[a]	>10[4]	ND[b]	ND
Cefepime	505 ± 68.7	67.7 ± 8.05	1.3×10^5

[a] Nitrocefin (100 μM) was used as the reporter substrate to obtain the K_is value.
[b] ND, not determined.

DISCUSSION

In this study, a novel chimeric β-lactamase, CTX-M-64, was first identified in a *S. sonnei* isolate recovered from a tourist who had returned from China. In Japan, approximately 80% of bacteriologically confirmed cases of shigellosis have been associated with tourists returning from foreign countries, especially from Asia, and *S. sonnei* has become the predominant cause of such enteric infections. Thus, it is important to monitor the emergence and the prevalence of the resistance mechanisms that may be introduced into the community by tourists who have traveled overseas and who are infected with enteropathogenic bacteria, including *Shigella* species.

In several survey studies, Woodford et al. (33) adopted multiplex PCR and Pitout et al. (23) adopted group-specific primers for the molecular classification of CTX-M-type β-lactamase genes. On the basis of the nucleotide sequence data of $bla_{CTX-M-64}$, PCR with the primer sets used by Pitout et al. (23) are expected to fail to produce any amplification product. On the other hand, the use of a combination of CTX-M-1-group-specific forward primer and a CTX-M-9-group-specific reverse primer (2-bp mismatch) by Woodford et al. (33) may well generate a 205-bp PCR product from the $bla_{CTX-M-64}$ gene, although a PCR product of this size would be indistinguishable from the PCR product generated from the $bla_{CTX-M-9-group}$ gene in their multiplex PCR system and would thus provide an incorrect result. Moreover, it appears to be certain that multiplex PCR methods are powerful tools for the classification of CTX-M-type β-lactamase genes, but hereafter, one will need to take into account the presence of hybrid-style β-lactamase genes like $bla_{CTX-M-64}$ when the preexisting multiplex PCR

fails to detect any of the known CTX-M-type β-lactamase genes.

CTX-M-64 showed high catalytic efficiencies against ampicillin, nitrocefin, cephalothin, and cefotaxime; and clavulanic acid behaved as a potent inhibitor of this enzyme. These enzymatic characteristics of CTX-M-64, as described above, are commonly observed in the majority of CTX-M-type enzymes. Additionally, CTX-M-64 had enhanced activity against ceftazidime, and this has been shown for a number of CTX-M-type enzymes. To evaluate further this property caused by CTX-M-64 production, we determined the kinetic parameters of CTX-M-64 against ceftazidime. Unfortunately, the catalytic efficiency (k_{cat}/K_m) of CTX-M-64 against ceftazidime could not be determined due to its very high K_i value. At present, two amino acid substitutions, Pro-167Ser and Asp-240Gly, have mainly been reported to be involved in the augmented hydrolytic activities of the CTX-M-type enzymes against ceftazidime (2, 12, 13, 24). Although the actual mechanism for the higher MIC of ceftazidime for CTX-M-64 producers remains uncertain, the glycine residue at position 240 in the CTX-M-64 enzyme probably plays a crucial role in the acquisition of the higher level of hydrolyzing activity against ceftazidime. In addition, it is speculated that the distinctive hybrid composition formed in CTX-M-64 might well cause particular steric interactions with ceftazidime and provide CTX-M-64 with its higher level of hydrolytic activity. Molecular modeling and X-ray crystallographic analyses would be needed to substantiate this speculation.

The $bla_{CTX\text{-}M\text{-}64}$ gene was flanked upstream by an IS*Ecp1* sequence and downstream by an *orf477* sequence. The presence of an IS*Ecp1* element upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene and an *orf477* element downstream of the $bla_{CTX\text{-}M\text{-}15}$ gene has been described previously (10), and IS*Ecp1* may contribute to the mobilization and high-level expression of the *bla* gene. Interestingly, the CTX-M-15-like enzyme has been identified in an *E. coli* clinical isolate in China, and the $bla_{CTX\text{-}M\text{-}64}$ gene as well as the $bla_{CTX\text{-}M\text{-}15\text{-}like}$ gene has been located 45 bp downstream from IS*Ecp1*, while the spacer region between IS*Ecp1* and $bla_{CTX\text{-}M\text{-}15}$ is generally 48 bp in length (10, 17, 24). Moreover, the spacer sequences of the $bla_{CTX\text{-}M\text{-}64}$ gene and the $bla_{CTX\text{-}M\text{-}15\text{-}like}$ gene shared two nucleotide substitutions from the corresponding sequence of the $bla_{CTX\text{-}M\text{-}15}$ gene, whereas they shared only one of these two nucleotide substitutions from the corresponding chromosomal $bla_{CTX\text{-}M\text{-}3}$ spacer sequence of *Kluyvera ascorbata* (GenBank accession no. AJ632119). The 391-bp region immediately downstream of the termination codon of the $bla_{CTX\text{-}M\text{-}64}$ gene showed 100% sequence identity to the corresponding region of the *K. ascorbata* chromosomal $bla_{CTX\text{-}M\text{-}3}$ (GenBank accession no. AJ632119), $bla_{CTX\text{-}M\text{-}3}$ (GenBank accession no. AF550415), and $bla_{CTX\text{-}M\text{-}15}$ (GenBank accession no. AY995206) genes. Moreover, the presence of a putative IRR of IS*Ecp1* described by Rodríguez et al. (26) at the right end of the 391-bp region is indicative of an IS*Ecp1*-mediated transposition event. Thus, the $bla_{CTX\text{-}M\text{-}15\text{-}like}$ gene might have originated from the $bla_{CTX\text{-}M\text{-}3}$ gene, which emerged by an independent mobilization event from the chromosome of a strain of *K. ascorbata* mediated by IS*Ecp1* inserted in its 45-bp upstream region (26). Then, the newly identified $bla_{CTX\text{-}M\text{-}64}$ gene might have emerged by a double-crossover-type homologous recombination event between the $bla_{CTX\text{-}M\text{-}15\text{-}like}$ gene located on the approximately 68-kb plasmid and the $bla_{CTX\text{-}M\text{-}14}$ gene possibly located on other plasmids coexisting in the same bacterial cell.

In conclusion, we report here on the emergence of the CTX-M-64 β-lactamase that shows a structure consisting of a chimera of two different CTX-M-type β-lactamase groups. In CTX-M-type β-lactamases, the acquisition of extended substrate specificity has so far been dependent on the accumulation of key amino acid substitutions that lead to changes in the steric interactions between the enzyme and the substrate agents (2, 12). Hereafter, however, it seems likely that the CTX-M-type β-lactamase would evolve to acquire the atypical substrate specificity through replacement of principal domains between cognate enzymes, as has been observed in CTX-M-64.

ACKNOWLEDGMENTS

We are grateful to Kunikazu Yamane, Naohiro Shibata, and Kouji Kimura, Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, for valuable scientific advice.

This work was supported by a grant (grant H18-Shinkou-011) from the Ministry of Health, Labor and Welfare of Japan.

We have no conflicts of interest.

REFERENCES

1. **Acikgoz, Z. C., Z. Gulay, M. Bicmen, S. Gocer, and S. Gamberzade.** 2003. CTX-M-3 extended-spectrum β-lactamases in a *Shigella sonnei* clinical isolate: first report from Turkey. Scand. J. Infect. Dis. **35**:503–505.
2. **Bonnet, R., C. Recule, R. Baraduc, C. Chanal, D. Sirot, C. De Champs, and J. Sirot.** 2003. Effect of D240G substitution in a novel ESBL CTX-M-27. J. Antimicrob. Chemother. **52**:29–35.
3. **Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey.** 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. Antimicrob. Agents Chemother. **46**:630–637.
4. **Christensen, W. B.** 1949. Hydrogen sulfide production and citrate utilization in the differentiation of enteric pathogens and coliform bacteria. Research bulletin no. 1. Weld County Health Department, Greeley, CO.
5. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A7, 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing. Eighteenth information supplement (M100-S18). Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Doi, Y., J. Wachino, M. Ishiguro, H. Kurokawa, K. Yamane, N. Shibata, K. Shibayama, K. Yokoyama, H. Kato, T. Yagi, and Y. Arakawa.** 2004. Inhibitor-sensitive AmpC β-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob. Agents Chemother. **48**:2652–2658.
8. **Dutour, C., R. Bonnet, H. Marchandin, M. Boye, C. Chanal, D. Sirot, and J. Sirot.** 2002. CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from *Enterobacteriaceae* isolated in France. Antimicrob. Agents Chemother. **46**:534–537.
9. **Dutta, S., D. Dutta, P. Dutta, S. Matsushita, S. K. Bhattacharya, and S.-I. Yoshida.** 2003. *Shigella dysenteriae* serotype 1: Kolkata, India. Emerg. Infect. Dis. **9**:1471–1474.
10. **Eckert, C., V. Gautier, and G. Arlet.** 2006. DNA sequence analysis of the genetic environment of various $bla_{CTX\text{-}M}$ genes. J. Antimicrob. Chemother. **57**:14–23.
11. **Kim, S., J. Kim, Y. Kang, Y. Park, and B. Lee.** 2004. Occurrence of extended-spectrum β-lactamases in members of the genus *Shigella* in the Republic of Korea. J. Clin. Microbiol. **42**:5264–5269.
12. **Kimura, S., M. Ishiguro, Y. Ishii, J. Alba, and K. Yamaguchi.** 2004. Role of a mutation at position 167 of CTX-M-19 in ceftazidime hydrolysis. Antimicrob. Agents Chemother. **48**:1454–1460.
13. **Kimura, S., Y. Ishii, K. Tateda, and K. Yamaguchi.** 2007. Predictive analysis of ceftazidime hydrolysis in CTX-M-type β-lactamase family members with a mutational substitution at position 167. Int. J. Antimicrob. Agents **29**:326–331.
14. **Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine.** 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation. Bull. W. H. O. **77**:651–656.
15. **Lartigue, M. F., L. Poirel, J. W. Decousser, and P. Nordmann.** 2005. Multidrug-resistant *Shigella sonnei* and *Salmonella enterica* serotype Typhi-