mixed Filipino-White heritage; both had IgG persistently detected beyond 12 months of age and were monitored clinically for retinochoroiditis. Their charts contained no information regarding maternal exposure or risk factors. During the 15-year period, the rate of diagnosed congenital toxoplasmosis was 3.8 (95% CI 1.5–9.2) per million live births. There were no infant deaths for which congenital toxoplasmosis was recorded as a cause. We were unable to study fetal deaths because the corresponding cause-of-death codes were not readily available.

Historically, the lowest prevalence of *T. gondii* infection has been recorded in the western United States (*5*). The rate of clinically apparent congenital toxoplasmosis in this study was lower than that found during the late 1980s through early 1990s in the New England Newborn Screening Program initially after birth (2 per 521,555 live births [3.8 per million] versus 5 per 635,000 live births [7.9 per million], respectively) (*6*). However, the prevalence of *T. gondii* infection has decreased in the United States since the 1990s (*1*).

Our study is subject to several limitations. Our approach would only detect clinically apparent cases, and the results should be considered a minimal estimate of congenital infection. Some cases may not have been recorded in the electronic system, but this omission is not likely for severe illness, repeated hospital or clinic visits, or outside consultation. The small number of cases makes the rate of diagnosed congenital toxoplasmosis somewhat imprecise; a few missed cases would increase the rate considerably. In addition, we were not able to evaluate fetal deaths; however, stillbirth is reportedly a rare complication of congenital toxoplasmosis (*7*). Although we found a low rate of diagnosed congenital toxoplasmosis in northern California, population-based studies to evaluate rates of the disease in other geographic areas would be beneficial.

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DOI: http://dx.doi.org/10.3201/eid2009.131919

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Invasive Infection Caused by Carbapenem-Resistant *Acinetobacter soli*, Japan

To the Editor: Infections caused by *Acinetobacter* spp., especially *A. baumannii*, have been increasingly documented in recent years. Carbapenems tend to be empirically prescribed as first-choice drugs for severe invasive infections caused by *Acinetobacter* spp. other than *A. baumannii* because these microbes are usually susceptible to carbapenems. However, infections with carbapenem-resistant *Acinetobacter* spp. have been increasingly reported during the past 15 years. In *A. baumannii*, carbapenems are usually inactivated by intrinsic oxacillinase (OXA)-51–like, acquired OXA-23–like, or OXA-58–like carbapenemases. Moreover, production of acquired metallo-β-lactamases (MBLs) of the Verona integron (VIM), imipenemase (IMP), or New Delhi (NDM) types has been detected among carbapenem-resistant *Acinetobacter* species, including *A. baumannii*, *A. junii*, *A. bereziniae*, *A. nosocomialis*, and *A. pittii* (*1*). We report a case of infection with carbapenem-resistant *A. soli* producing another

MBL type, Tripoli MBL 2 (TMB-2), in a man in Japan.

A man in his 60s who had mesenteric injury, pelvic fracture, and intestinal perforation from a traffic accident was admitted to Okazaki City Hospital in Aichi, Japan, on May 3, 2013. After surgery, cefmetazole was prescribed on May 6 (1 g 2×/d for 7 d). On May 12, symptoms of infection developed in the patient, and 2 sets of blood samples were drawn from different vessels for bacterial culture. The following day, cefmetazole was discontinued, and ciprofloxacin (0.3 g 2×/d) and piperacillin/tazobactam (4.5 g 2×/d) were started. *Acinetobacter* isolates resistant to piperacillin/tazobactam and carbapenems were then recovered from the blood samples, so piperacillin/tazobactam was discontinued on May 14. After that, ceftriaxone (2 g 2×/d) and gentamicin (0.04 g 2×/d) were successively prescribed, in addition to ciprofloxacin; the symptoms of infection improved, and all antimicrobial drugs were discontinued by May 26. Additional blood cultures performed on May 17, 21, and 28 yielded negative results for *Acinetobacter* spp. However, the patient's condition worsened on June 5. Meropenem (0.5 g 4×/d) was then given, but the patient died of multiorgan failure on June 7.

The bacterial isolates from the initial blood cultures were identified as *A. soli* by nucleotide sequencing of the *rpoB* and *gyrB* genes and assigned identification no. HK001. MICs of β-lactams, measured by the agar dilution method in accordance with the guideline M07-A9 of the Clinical and Laboratory Standards Institute (http://clsi.org), were as follows: sulbactam/ampicillin, >128 mg/L; piperacillin, >128 mg/L; tazobactam/piperacillin, >128 mg/L; cefotaxime, >64 mg/L; ceftazidime, >64 mg/L; aztreonam, 64 mg/L; cefmetazole, >128 mg/L; imipenem, 8 mg/L; meropenem, 32 mg/L; and doripenem, 32 mg/L. However, MICs of gentamicin, amikacin, levofloxacin, ciprofloxacin, colistin, and tigecycline were below the breakpoints of susceptibility as listed in Clinical and Laboratory Standards Institute document M100-S23. Carbapenem resistance was not transferred from *A. soli* HK001 to *Escherichia coli* strain CSH-2 (*metB* F^- NA^r Rif^r) by conjugation. A double-disk synergy test was initially performed by using sodium mercaptoacetic acid (SMA) (*2*) and ceftazidime and meropenem disks (Eiken Chemical Co., Ltd, Tokyo, Japan), and results suggested MBL production. The modified Hodge test was then performed, and ertapenem and meropenem disks gave clear positive results (data not shown). PCR was performed to detect bla_{OXA-23}–like, $bla_{OXA-24/40}$–like, bla_{OXA-51}–like, bla_{OXA-58}–like, bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1}, bla_{VIM-2}, bla_{NDM-1}, bla_{SMB-1}, and bla_{TMB-1} genes. Nucleotide sequence analyses showed that the *A. soli* isolate harbored bla_{TMB-2} and bla_{OXA-58}. The modified SMA-disk method (*3*) was reevaluated to determine whether it could successfully detect TMB-2 production in *A. soli* HK001. Apparent positive results were obtained when disks containing imipenem, meropenem, or ertapenem were used, particularly when the edge-to-edge distance between 2 disks containing SMA and a carbapenem, respectively, was kept at 5 mm (Figure, top row). However, when the distance between the ertapenem and SMA disks was ≥10 mm, MBL production was more difficult to detect (Figure, lower 2 rows). This finding may be the result of co-production of OXA-58 by the isolate.

More than 30 *Acinetobacter* species had been registered by January 2012 (*4*); *A. soli* was initially isolated from the soil of a mountain forest in South Korea in 2007 (*5*) and has been recovered from blood cultures of 5 neonates in Brazil (*6*). Carbapenem-resistant *A. soli* co-harboring bla_{IMP-1} and bla_{OXA-58}–like genes was identified in April 2011 in Japan and is frequently recovered from bacteremia patients (*7*). TMB-1 was reported in 2012 in an *Achromobacter xylosoxidans* isolate from a hospital in Tripoli, Libya (*8*); TMB-2 was later reported in Japan

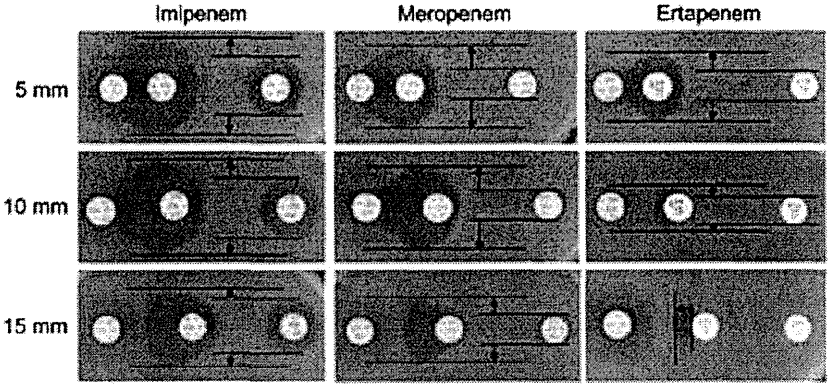

Figure. Results of double-disk synergy testing of the *Acinetobacter soli* isolate HK001 identified in a man in Japan. Testing was performed by using disks containing sodium mercaptoacetic acid (SMA) and the carbapenems imipenem, meropenem, and ertapenem. Apparent expansion of growth inhibition zone around a carbapenem disk placed near a SMA disk compared with that around a disk of carbapenem alone is seen on Mueller-Hinton agar if the isolate produces metallo-β-lactamases (*2*,*3*). When the edge-to-edge distance between 2 disks containing a carbapenem and SMA, respectively, was kept at 5 mm, expansion of the growth inhibition zone became clearer than for those kept at a distance of 10 mm and 15 mm, regardless of carbapenems used. Vertical expansion of growth inhibition zones by the effect of SMA is indicated by arrows; ertapenem gave the clearest result when the disk distance was kept at 5 mm (top right panel), even though *A. soli* HK001 co-produces oxacillinase 58–like carbapenemase, which is hardly inhibited by SMA.

(*9*). The TMB-2–producing *A. soli* strain that we isolated came from a blood culture, indicating that *A. soli* is a potential cause of bloodstream infections or bacteremia. *A. soli* has also been detected in lice and keds of domestic animals (*10*), indicating that *A. soli* may inhabit natural environments and that injuries and bites by arthropods might present a risk for invasive infections. Isolates of *Acinetobacter* species, particularly those recovered from blood culture, should be identified to species type to enable further evaluation of the clinical significance of carbapenem-resistant *A. soli* strains.

This study was supported by the Ministry of Health, Labour and Welfare, Japan (H25-Shinko-Ippan-003 and H24-Shinko-Ippan-010).

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DOI: http://dx.doi.org/10.3201/eid2009.140117

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Spread of Vaccinia Virus to Cattle Herds, Argentina, 2011

To the Editor: Since 1999, several zoonotic outbreaks of vaccinia virus (VACV) infection have been reported in cattle and humans in rural areas of Brazil. The infections have caused exanthematous lesions on cows and persons who milk them, and thus are detrimental to the milk industry and public health services (*1*,*2*). In Brazil during the last decade, VACV outbreaks have been detected from the north to the extreme south of the country (*1*–*4*). Because Brazil shares extensive boundaries with other South American countries, humans and cattle on dairy and beef-producing farms in those countries may be at risk of exposure to VACV. To determine if VACV has spread from Brazil to Argentina, we investigated the presence of VACV in serum samples from cattle in Argentina.

During 2011, we obtained serum samples from 100 animals (50 dairy and 50 beef cattle) on farms in Córdoba, Corrientes, Entre Ríos, and Santa Fe Provinces in Argentina (online Technical Appendix, panel A, http://wwwnc.cdc.gov/EID/article/20/9/14-0154-Techapp1.pdf). No VACV cases had been reported in humans or cattle in these provinces. However, Corrientes Province borders the Brazilian state of Rio Grande do Sul, where VACVs (Pelotas 1 and Pelotas 2 viruses) were isolated during an outbreak affecting horses in 2008 (*2*).

To determine the presence of neutralizing antibodies in the serum samples, we used an orthopoxvirus 70% plaque-reduction neutralization test as described (*4*). On the basis of previous studies that detected viral DNA in serum samples (*4*–*6*), we used real-time PCR to amplify the highly conserved orthopoxvirus vaccinia growth factor (*vgf*) gene DNA (P.A. Alves, unpub. methods).

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Evaluation of Disk Potentiation Test Using Kirby-Bauer Disks Containing High-Dosage Fosfomycin and Glucose-6-Phosphate To Detect Production of Glutathione *S*-Transferase Responsible for Fosfomycin Resistance

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The main cause of fosfomycin resistance in extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates is glutathione *S*-transferase responsible for fosfomycin resistance (FR-GST), which includes FosA3, FosA4, and FosC2 (1, 2). Fosfomycin is expected to become an option for treating urinary tract infections caused by ESBL-producing *E. coli* (3), but FR-GST genes have already spread among *E. coli* isolates in a variety of clinical and veterinary settings, which limits the potential use of fosfomycin (1, 4–7). Detection of FR-GST-producing bacteria by clinical microbiology laboratories is, therefore, necessary to prevent their further spread in clinical settings. To easily identify the production of FR-GST, we have recently developed a simple, cost-effective disk potentiation test, using phosphonoformate (PPF), a specific inhibitor of FR-GST (2, 8). The test uses Kirby-Bauer (KB) disks containing 50 μg fosfomycin and 5 μg glucose-6-phosphate (G6P), in combination with Mueller-Hinton (MH) agar plates containing 25 μg/ml G6P (MH-G6P plates). The addition of G6P to the agar plates increased the inhibitory effect by PPF, compared to MH plates without G6P supplementation, enabling unambiguous identification of FR-GST production (2).

Clinical and Laboratory Standards Institute (CLSI) guidelines recommend the use of KB disks containing 200 μg fosfomycin and 50 μg G6P when performing disk diffusion susceptibility testing (9). These amounts are 4- and 10-fold higher, respectively, than those we have used (2). In this study, we have incorporated the high-dosage fosfomycin-G6P KB disks into the disk potentiation test to evaluate their effectiveness in screening for FR-GST production.

Fifteen FR-GST-positive *E. coli* isolates (12 isolates with *fosA3*, 2 isolates with *fosA4*, and 1 isolate with *fosC2*) and 22 FR-GST-negative *E. coli* isolates were used for the evaluation (2). The strains tested were prepared according to the CLSI guidelines (9) and spread on MH plates and MH-G6P plates. Two blank disks were placed on the agar plates; the first disk was loaded with 200 μg fosfomycin and 50 μg G6P (20 μl was loaded with the solution containing 10-mg/ml fosfomycin and 2.5-mg/ml G6P dissolved in water), and the second disk was additionally loaded with 1 mg PPF (Fig. 1). After 18 h of incubation at 37°C, the growth inhibitory zone around each disk was measured, and the results were summarized in Fig. 2. Fifteen FR-GST-positive isolates exhibited 11- to 19-mm (average 14-mm) expansion and 10- to 14-mm (average 12-mm) expansion in the growth inhibition zone by the

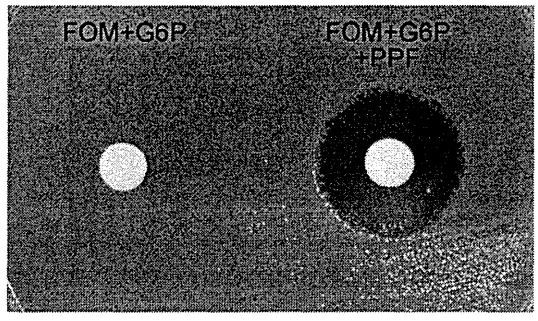

FIG 1 Potentiation test for a FosA3-producing *E. coli* isolate on an MH plate. The left disk contains 200 μg fosfomycin (FOM) and 50 μg glucose-6-phosphate (G6P), and the right disk contains 200 μg fosfomycin, 50 μg G6P, and 1 mg phosphonoformate (PPF).

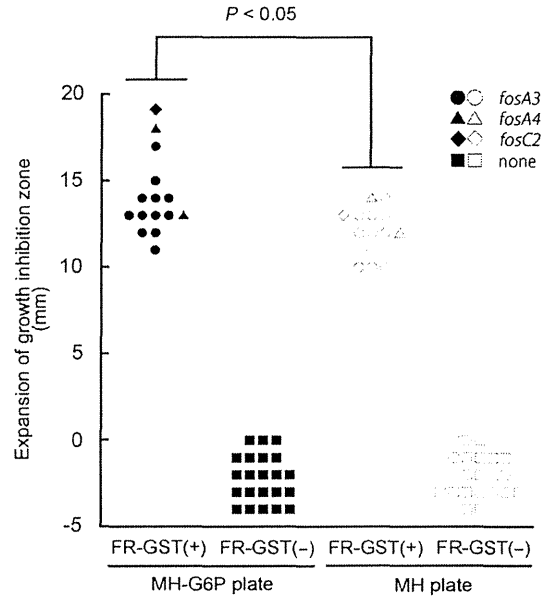

FIG 2 Changes to growth inhibition zone diameter by PPF on MH-G6P and MH plates. The *y* axis shows enlargement of the growth inhibition zone (in millimeters) by PPF. FR-GST-positive [FR-GST(+)] and FR-GST-negative [FR-GST(−)] isolates are shown.

Published ahead of print 13 August 2014
Editor: R. Patel
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doi:10.1128/JCM.01805-14

addition of PPF on MH-G6P and MH plates, respectively ($P < 0.05$ by the Wilcoxon signed-rank test). When the FR-GST-positive strains were grown on MH-G6P plates, the growth inhibition zone was larger than that on MH plates. Thus, the addition of G6P to MH plates resulted in a larger inhibitory zone, confirming the results of our previous study (2). However, MH plates without the addition of G6P resulted in enough growth inhibition zone expansion to enable the detection of FR-GST production (Fig. 2). The FR-GST-negative strains showed no growth inhibition zone expansion by PPF, although a slight reduction in the size of the zone was observed, as shown by our previous study (2). Therefore, the use of high-dosage fosfomycin-G6P disks containing 200 μg fosfomycin and 50 μg G6P could eliminate the need for additional G6P supplementation in the potentiation test for identifying FR-GST producers.

Together with our previous results (2), we confirmed that both the Western standard disks (high-dosage fosfomycin-G6P) with MH plates and Japanese standard disks (low-dosage fosfomycin-G6P) with MH plates supplemented with 25 μg/ml G6P can detect FR-GST-producing *E. coli* isolates.

ACKNOWLEDGMENTS

This study was supported by grants from the Japanese Ministry of Health, Labour and Welfare (H24-Shinkou-Ippan-010).

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Practical Agar-Based Disk Potentiation Test for Detection of Fosfomycin-Nonsusceptible *Escherichia coli* Clinical Isolates Producing Glutathione *S*-Transferases

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The number of reports concerning *Escherichia coli* clinical isolates that produce glutathione *S*-transferases responsible for fosfomycin resistance (FR-GSTs) has been increasing. We have developed a disk-based potentiation test in which FR-GST producers expand the growth inhibition zone around a Kirby-Bauer disk containing fosfomycin in combination with sodium phosphonoformate (PPF). PPF, an analog of fosfomycin, is a transition-state inhibitor of $FosA^{PA}$, a type of FR-GST from *Pseudomonas aeruginosa*. Considering its mechanism of action, PPF was expected to inhibit a variety of FR-GSTs. In the presence of PPF, zone enlargement around the disk containing fosfomycin was observed for FosA3-, FosA4-, and FosC2-producing *E. coli* clinical isolates. Moreover, the growth inhibition zone was remarkably enlarged when the Mueller-Hinton (MH) agar plate contained 25 μg/ml glucose-6-phosphate (G6P). When we retrospectively tested 12 fosfomycin-resistant (MIC, ≥256 μg/ml) *E. coli* clinical isolates from our hospital with the potentiation test, 6 FR-GST producers were positive phenotypically by potentiation disk and were positive for FR-GST genes: 5 harbored *fosA3* and 1 harbored *fosA4*. To identify the production of FR-GSTs, we set the provisional cutoff value, 5-mm enlargement, by adding PPF to a fosfomycin disk on the MH agar plates containing G6P. Our disk-based potentiation test reliably identifies FR-GST producers and can be performed easily; therefore, it will be advantageous in epidemiological surveys and infection control of fosfomycin-resistant bacteria in clinical settings.

The increased emergence of multidrug-resistant (MDR) pathogenic bacteria is becoming a serious public health concern, as MDR bacteria limit the choice of antimicrobials available for treatment (1). In such a situation, "old" antimicrobials, such as colistin and fosfomycin, have been reintroduced into clinical practice to overcome the difficulties posed by MDR pathogens (2). However, some clinically isolated bacteria have already developed resistance to these reintroduced antimicrobials (3, 4). We have previously demonstrated that CTX-M-type extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates have already acquired resistance to fosfomycin, although the incidence of resistance is still low (3.6%) (5). In addition, we revealed that some ESBL-producing fosfomycin-resistant *E. coli* clinical isolates can inactivate fosfomycin by producing glutathione *S*-transferases (GSTs), such as FosA3 and FosC2 (Fig. 1) (5). These fosfomycin resistance determinants were located on transferable plasmids and were mostly linked with CTX-M-type ESBL genes (5, 6).

Since our first report of fosfomycin-resistant *E. coli* organisms producing FosA3 in 2010, reports from East Asian countries have identified many FosA3 producers in *E. coli* isolates from clinical specimens (7), healthy individuals (6), consumable animal products (8, 9), and domestic animals (10). Further spread of the gene encoding FosA3 would be a serious public health concern because of the global distribution of CTX-M-type ESBL producers in a variety of settings (11).

To identify FosA3-producing *E. coli* organisms and prevent their further spread, it is important to develop a specific detection method. PCR is the most common technique used to detect specific antibiotic resistance determinants, but its availability is generally limited to highly advanced facilities, such as research laboratories and university hospitals. Hence, a simple yet cost-effective method, such as the ESBL confirmation test, which uses commercially available antibiotic disks in combination with a potent inhibitor, would be a preferred tool for screening bacteria with specific antibiotic resistance mechanisms in clinical microbiology laboratories (12). The aim of this study was to develop a simple and cost-effective detection method based on the standard disk diffusion test in order to identify *E. coli* isolates producing fosfomycin resistance-mediating glutathione *S*-transferases (FR-GSTs), such as FosA3.

MATERIALS AND METHODS

Bacterial strains. A total of 25 *E. coli* clinical isolates from our laboratory stock, including seven *fosA3*-positive isolates, one *fosC2*-positive isolate, one *fosA4*-positive isolate, and 16 isolates without any of these three genes, were used to collect basic data to develop the disk-based potentiation test described below. All the isolates were nonsusceptible to fosfomycin (MIC, ≥128 μg/ml). Detailed characterizations were reported for some of these isolates in our previous studies (5, 6).

PCR. The presence of the FR-GST genes, *fosA3*, *fosA4*, and *fosC2*, was confirmed by PCR using the primer sets described in Table 1. The detailed

Received 14 April 2014 Returned for modification 20 May 2014
Accepted 6 June 2014
Published ahead of print 20 June 2014
Editor: R. Patel
Address correspondence to Jun-ichi Wachino, wachino@med.nagoya-u.ac.jp.

doi:10.1128/JCM.01094-14

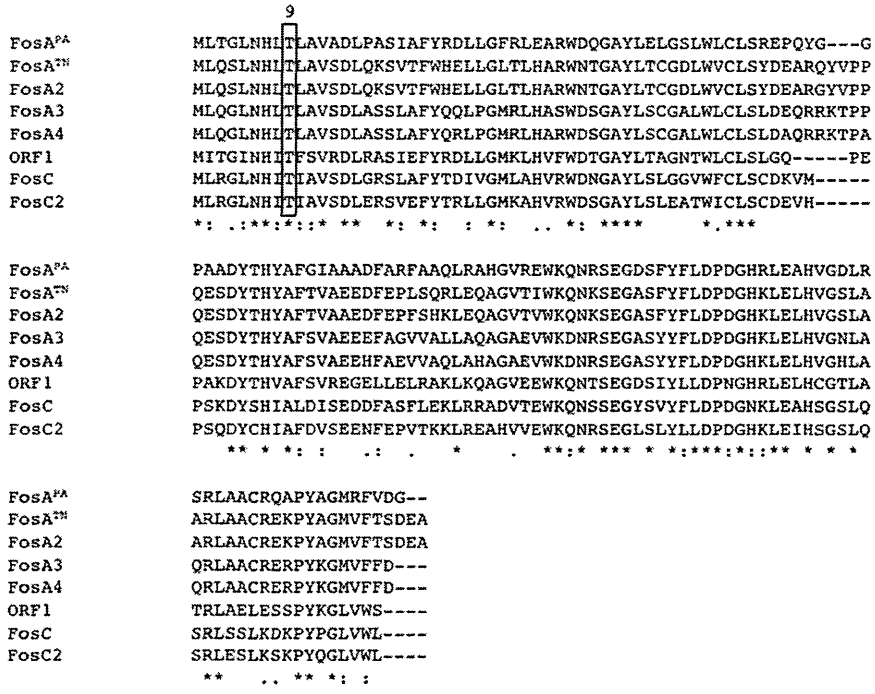

FIG 1 Predicted amino acid sequence of fosfomycin resistance determinants. *, amino acid residues conserved among the eight fosfomycin resistance determinants; colons and dots, amino acid substitutions that result in homologous amino acid residues. The resistance determinants (GenBank accession no.) are $FosA^{PA}$ (AAT49669), $FosA^{TN}$ (AAA98399), FosA2 (ACC85616), FosA3 (BAJ10054), FosA4 (AB908992), open reading frame 1 (ORF1) (AAP50248), FosC (AAZ14834), and FosC2 (BAJ10053). The box indicates the conserved Thr9 residue in the fosfomycin resistance glutathione *S*-transferases.

genotypes of *fosA3*/*fosA4* were determined by PCR and nucleotide sequencing analyses using primers designed on the basis of the sequences deposited in GenBank (accession no. AB522970) (Table 1). The PCR conditions were 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min.

Susceptibility testing. The MIC of fosfomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for each strain was determined using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (13). *E. coli* strain ATCC 25922 was used as the control.

Disk potentiation test. The *E. coli* isolates were adjusted to a McFarland standard of 0.5 according to the CLSI guideline and inoculated onto a Mueller-Hinton (MH) agar plate (Nippon Becton, Dickinson Co., Ltd., Tokyo, Japan) and an MH agar plate containing 25 μg/ml glucose-6-phosphate (G6P) (MH-G6P plate) (Sigma-Aldrich, St. Louis, MO). The addition of G6P to the MH agar plates is specified in the CLSI guidelines when determining the MIC of fosfomycin for *E. coli* (12). Two Kirby-Bauer (KB) disks containing fosfomycin (50 μg/disk) and G6P (5 μg/disk) (Eiken Chemical Co., Ltd., Tokyo, Japan) were placed on the plates. Sodium phosphonoformate (PPF) (Sigma-Aldrich), a candidate inhibitor of FR-GSTs, was dissolved in water to a concentration of 50 mg/ml. The inhibitor (20 μl, 1 mg in total) was applied to one of the two fosfomycin disks and incubated for 18 h at 37°C, and the diameter of the growth inhibition zone (i.e., the area where bacterial growth was completely inhibited) around each disk was measured. The diameter of the growth inhibition zone around a fosfomycin disk with PPF was compared with that around a fosfomycin disk. To test the antibacterial activity of sodium PPF itself, PPF (1 mg) was added to a blank disk and incubated, and the inhibition zone was measured.

Retrospective screening with the potentiation test. Sixteen *E. coli* clinical isolates that had been predicted to be ESBL producers (cefotaxime MIC, >2 μg/ml) with MIC values that were >16 μg/ml for fosfomycin were selected from the *E. coli* laboratory collection stocked in the clinical microbiology laboratory of Nagoya University hospital (a 1,000-bed tertiary care national university hospital). The characteristics of these bacterial isolates were preliminarily determined using the MicroScan WalkAway system (Siemens Healthcare Diagnostics, Tokyo, Japan) as part of the routine microbiology laboratory workup. These 16 isolates were subjected to fosfomycin susceptibility testing using the agar dilution method. Those isolates that were found to be nonsusceptible to fosfomycin (MIC, ≥128 μg/ml) were further subjected to the disk potentiation test and genetic characterization for genes mediating fosfomycin resistance.

Nucleotide sequence accession number. The nucleotide sequence of *fosA4* has been recorded in GenBank under accession no. AB908992.

TABLE 1 Primers used in this study

Primer	Sequence
fosA3/4-forward	5′-TGA ATC ATC TGA CGC TGG-3′
fosA3/4-reverse	5′-TCA ATC AAA AAA GAC CAT C-3′
fosC2-forward	5′-CGT TCC GTG GAG TTC TAT AC-3′
fosC2-reverse	5′-CTT GAT AGG GTT TAG ACT TC-3′
fosA3/4En-forward	5′-CGA TCA CAG TTT ACA ACA GG-3′
fosA3/4En-reverse	5′-GGC TAT CTT GCT CAG CTC TA-3′

RESULTS

In the present study, we developed a practical disk potentiation test to macroscopically detect the production of FR-GSTs in bacterial cells using PPF. The results for two representative *E. coli* isolates, one *fosA3* positive and the other FR-GST negative, are shown in Fig. 2A. Both strains showed resistance to fosfomycin (MIC, ≥512 μg/ml), resulting in almost no growth inhibition zone around the fosfomycin disks. Following the addition of PPF, a 4-mm expansion of the growth inhibition zone around the fos-

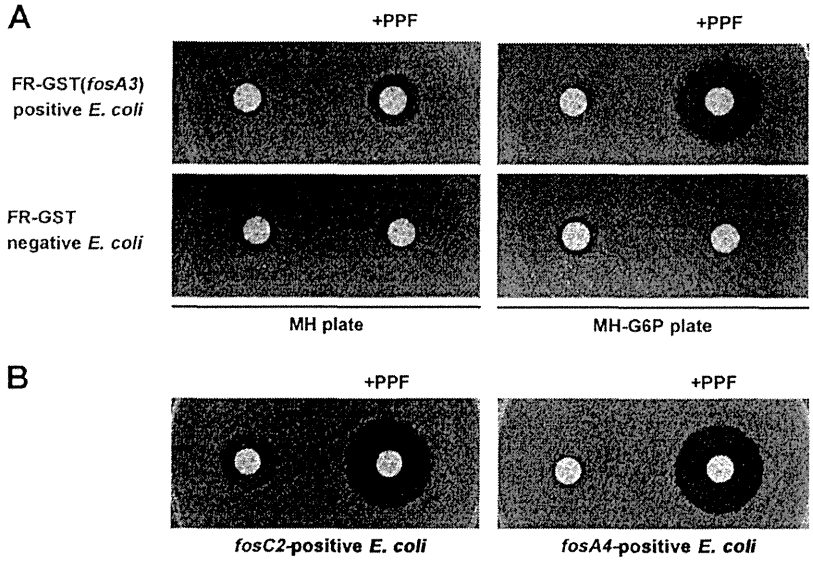

FIG 2 (A) Potentiation by PPF in growth inhibition zone diameters around a fosfomycin disk. PPF was added to a fosfomycin disk (right side in each panel). Shown are a *fosA3*-positive strain on an MH plate (upper left) and MH-G6P plate (upper right), and an FR-GST-negative strain on an MH plate (lower left) and MH-G6P plate (lower right). (B) The results of *fosC2*-positive (left) and *fosA4*-positive strains on MH-G6P plates (right). FR-GST, fosfomycin resistance-mediating glutathione *S*-transferase; PPF, phosphonoformate; MH, Mueller-Hinton; G6P, glucose-6-phosphate.

fomycin disk on the MH agar plate was observed for the *fosA3*-positive strain (Fig. 2A, upper left), while a 13-mm-greater expansion of the growth inhibition zone was found on the MH-G6P plate (Fig. 2A, upper right). The addition of G6P to the MH agar plate resulted in the formation of larger growth inhibition zones around the fosfomycin disks. For a fosfomycin-resistant strain not harboring any FR-GST genes, no zone enlargement was observed with PPF; however, in fact, a slight reduction in the size of the zone was observed on both the MH and MH-G6P plates (Fig. 2A, lower left and right). To test the antibacterial activity of PPF alone, 1 mg PPF was added to a blank disk. For both strains, no growth inhibition zone was observed around the PPF disk, indicating that PPF by itself had no apparent suppressive effect on bacterial growth (data not shown). Taken together, the expansion of the growth inhibition zone by PPF, especially on MH-G6P plates, is a good indicator of FR-GST production, probably via the inactivation of FR-GST activity.

To confirm the reliability of the developed method, we tested the remaining 23 fosfomycin-intermediate and -resistant *E. coli* isolates from our laboratory stock (6 *fosA3*-positive, 1 *fosC2*-positive, 1 *fosA4*-positive, and 15 isolates that were negative for these three genes), except the two representative strains shown in Fig. 2A. The *fosA3*-positive fosfomycin-resistant isolates showed an enlargement of the growth inhibition zone of 11 to 14 mm around the fosfomycin disk containing PPF on MH-G6P plates (Fig. 3). The fosfomycin-intermediate and -resistant (MIC range, 128 to ≥512 μg/ml) isolates not harboring any FR-GST genes showed either no increase or a slight decrease in diameter (up to 4 mm) on MH-G6P plates (Fig. 3), although it is unclear why a slight reduction in zone diameter was observed with PPF. Compared with the MH plates, a considerable expansion of the growth inhibition zones on the MH-G6P plates was observed for all *fosA3*-positive strains (Fig. 3). Thus, MH-G6P agar plates are useful for identifying FosA3 production. This disk potentiation test, which uses PPF as an inhibitor of FR-GST and MH-G6P plates, makes it possible to easily identify FosA3-producing strains among fosfomycin-intermediate and -resistant *E. coli* clinical isolates with high reliability.

We applied our method to a *fosC2*-positive *E. coli* isolate (5) and observed an 11-mm expansion of the growth inhibition zone (Fig. 2B, left). In addition, an expansion of the growth inhibition zone (15 mm) was observed for one *E. coli* isolate producing FosA4 (Fig. 2B, right), which is a newly identified variant of FosA3; FosA4 shares 94% amino acid identity with FosA3 (Fig. 1).

Finally, to validate whether our test works well for identifying FR-GST producers, we retrospectively screened 16 *E. coli* clinical isolates that were preliminarily classified as probable ESBL pro-

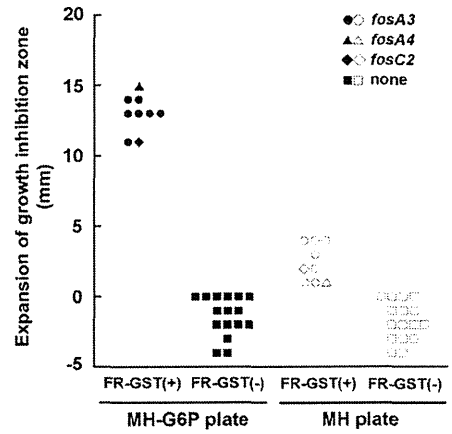

FIG 3 Summary of changes in growth inhibition zone diameter with PPF. The *y* axis represents the enlargement of the growth inhibition zone (mm) by PPF. FR-GST, fosfomycin resistance-mediating glutathione *S*-transferase; PPF, phosphonoformate; MH, Mueller-Hinton; G6P, glucose-6-phosphate.

ducers, with an MIC of >16 μg/ml for fosfomycin, in our clinical microbiology laboratory. Among these 16 isolates, 12 were fosfomycin resistant (MIC, ≥256 μg/ml) and 4 were fosfomycin susceptible (MIC, ≤64 μg/ml). Thus, 12 fosfomycin-resistant isolates were subjected to the disk-based potentiation test. We observed that six isolates exhibited an expansion of their growth inhibition zones (7 to 13 mm) with PPF, but the remaining six isolates showed no enlargement of their growth inhibition zones with PPF. PCR and sequencing analyses were performed to confirm the presence of FR-GST genes in the 12 isolates. The six isolates that showed positive results in the disk potentiation test also gave positive results for FR-GST genes: 5 harbored *fosA3* and 1 harbored *fosA4*. The remaining six isolates that showed negative results in the potentiation test were also negative for the *fosA3*, *fosA4*, and *fosC2* genes.

DISCUSSION

Rigsby et al. (14) previously demonstrated the crystal structure of an FR-GST, namely, $FosA^{PA}$ (PA1129) from *Pseudomonas aeruginosa*, in a complex with PPF (14). PPF bound to $FosA^{PA}$ via interaction with MnII(+) and Thr9 at the active site of $FosA^{PA}$ and behaved as a transition-state inhibitor analogous to fosfomycin. Notably, the Thr9 residue is conserved among all types of FR-GSTs (Fig. 1), including FosA3, which is active in the presence of MnII(+), similar to $FosA^{PA}$ (5, 15). Therefore, we hypothesize that PPF may be an effective inhibitor of not only $FosA^{PA}$ but also other FR-GSTs, such as FosA3, FosA4, and FosC2. We then used this agent to develop a practical disk potentiation test for detecting FR-GST producers. In addition, we found that supplementing the agar plates with G6P (Fig. 2 and 3) enabled us to obtain definitive results from our potentiation test. Although the commercially available fosfomycin disks in Japan contain G6P (5 μg/disk), adding G6P to the agar plates used in the test enhances the inhibitory effects caused by PPF. The addition of G6P may have accelerated the influx of fosfomycin into the bacterial cells by stimulating the expression of the UhpT transporter (16). In a disk diffusion test, the CLSI guidelines recommend using standardized fosfomycin disks containing 200 μg fosfomycin and 50 μg G6P, which are 4- and 10-fold higher, respectively, than the amounts used in this study. The use of disks containing such high dosages, especially of G6P, may eliminate the need to add G6P to the MH agar plates in our potentiation test. Nonetheless, in this study, the use of MH-G6P plates and disks containing 50 μg fosfomycin enabled us to reliably identify FR-GST producers among fosfomycin-nonsusceptible *E. coli* clinical isolates.

PPF seems to be a common inhibitor of all types of FR-GSTs known to date, which generally retain the Thr9 residue and are probably active in the presence of MnII(+), as demonstrated by the resistance determinants FosA3 and $FosA^{PA}$ (5, 15) (Fig. 1). Thus, our test appears to be capable of identifying any type of FR-GSTs, as shown in Fig. 1, in which the active site contains the key residue Thr9 and MnII(+), and of successfully detecting the production of FosA3, FosA4, and FosC2. However, because the number of the isolates used in the present study was limited, further evaluation will be required to determine whether our test can be used to identify all types of FR-GST producers. Recently, it was reported that the *fosA3* gene has been detected in *Klebsiella pneumoniae* clinical isolates (7). Future studies are required to evaluate the capability of the test developed here to detect FR-GST producers other than *E. coli*.

To identify FR-GST production, we established a 5-mm enlargement as the provisional cutoff value for the expansion of the diameter of the zone around the fosfomycin disk containing PPF on an MH-G6P plate compared with the diameter of the growth inhibition zone around the disk containing fosfomycin alone. This cutoff value was determined by taking the average minus 3 standard deviations of the values obtained for the changes in the diameter of the growth inhibition zones for 15 FR-GST-positive strains analyzed in this study. Using our cutoff value, the sensitivity and specificity of our test for identifying FR-GST producers were both 100%.

In this study, we used the potentiation test that we had developed to retrospectively screen for FR-GST producers among *E. coli* clinical isolates whose susceptibility to fosfomycin had preliminarily been determined through an automated system during routine work in the clinical microbiology laboratory. Using this test, we were able to classify FR-GST producers among the fosfomycin-resistant isolates. Although the mechanisms underlying fosfomycin resistance in those isolates that gave negative results in the potentiation test have not been fully characterized in the present study, the resistance of these isolates to fosfomycin does not depend on the production of FR-GSTs. Our test is simple and cost-effective; thus, it can be incorporated into the practice of screening for FR-GST producers in clinical microbiology laboratories.

In conclusion, our new method is simple, highly sensitive, specific, and feasible for routine use in clinical microbiology laboratories for identifying fosfomycin-resistant *E. coli* isolates producing FR-GSTs, such as FosA3, FosA4, and FosC2. Because FosA3-producing *E. coli* organisms have been identified in food animals and pets in China (8–10), fosfomycin-resistant *E. coli* should be assessed using the potentiation test in order to identify FR-GST-positive microbes in livestock farming environments at the early stage of emergence. We believe that our test will contribute significantly to both epidemiological analyses and better infection controls of fosfomycin-resistant bacteria in clinical settings.

ACKNOWLEDGMENTS

This study was supported by grants from the Japanese Ministry of Health, Labour, and Welfare (H24-Shinkou-Ippan-010 and H25-Shinkou-Ippan-003).

We thank the clinical microbiology laboratory staff for providing the bacterial isolates used in this study.

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Penicillin-Susceptible Group B Streptococcal Clinical Isolates with Reduced Cephalosporin Susceptibility

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We characterized penicillin-susceptible group B streptococcal (PSGBS) clinical isolates exhibiting no growth inhibition zone around a ceftibuten disk (CTB^r PSGBS). The CTB^r PSGBS isolates, for which augmented MICs of cefaclor and ceftizoxime were found, shared a T394A substitution in penicillin-binding protein 2X (PBP 2X) and a T567I substitution in PBP 2B, together with an additional G429S substitution in PBP 2X or a T145A substitution in PBP 1A, although the T145A substitution in the transglycosidase domain of PBP 1A would have no effect on the level of resistance to ceftibuten.

Group B streptococcus (GBS) is one of the principal causes of neonatal sepsis and meningitis. GBS also causes cutaneous and invasive infections in adults, including pregnant women, elderly people, and immunocompromised individuals. Serotypes III and Ia are predominant in isolates from neonates with invasive infections (1, 2). In adults with invasive infections, the most common serotype reported in Japan is Ib, but this serotype is not frequent in other countries (1, 3–5). Penicillin is the first-line antibiotic for treating GBS disease and for intrapartum chemoprophylaxis. We recently reported GBS isolates with reduced penicillin susceptibility (PRGBS), where at least two key amino acid substitutions, V405A and/or Q557E, in penicillin-binding protein 2X (PBP 2X) contribute to a considerable reduction in β-lactam susceptibility (6). Besides these two key substitutions, multiple amino acid substitutions were also found in PBP 2X, PBP 2B, and PBP 1A among PRGBS isolates, depending on the penicillin MIC levels (6–8). After our aforementioned study, PRGBS isolates harboring amino acid substitutions in PBPs were also reported in the United States and Canada (9–11). The prevalence of penicillin nonsusceptibility among GBS isolates from various clinical sources has increased from around 4.5% between 2007 and 2012 to 6.6% in 2013, according to the Japan Nosocomial Infections Surveillance (JANIS) of the Ministry of Health, Labour and Welfare (see http://www.nih-janis.jp). Since a low to moderate increase in penicillin MICs for PRGBS isolates has been observed, it is still very difficult to distinguish them from penicillin-susceptible GBS (PSGBS) isolates in routine susceptibility tests using disk diffusion or microdilution methods (12). However, a reduction in growth inhibition zone diameters or an elevation of the MICs of ceftizoxime and ceftibuten was found to be a good marker for screening of PRGBS (6, 13). Most GBS isolates displaying no growth inhibition zone around a ceftibuten disk were identified to be PRGBS isolates. However, we occasionally noticed a very small number of PSGBS isolates that exhibited no growth inhibition zone around the ceftibuten disk (CTB^r PSGBS). The objective of the present study was to characterize these CTB^r PSGBS isolates for a better understanding of the mechanisms underlying the reduced susceptibility to cephalosporins and penicillins in GBS.

In May 2011 and January 2012, six clinical isolates of CTB^r PSGBS, B1 to B6, were isolated clinically in a general hospital located in Chiba Prefecture, Japan. All of those isolates were serotype Ib, so an additional two PRGBS isolates (A1 and A2) and a PSGBS isolate (B7) with serotype Ib were selected for comparative analyses (Table 1). The diameters of the growth inhibition zones produced around disks containing 30 μg of ceftibuten per disk (6.35-mm diameter; Eiken Chemical Co., Ltd., Tokyo, Japan) were measured by Kirby-Bauer's disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (13, 14). MICs were determined by a broth microdilution method and were interpreted by following CLSI guidelines (14, 15). Ceftizoxime MICs were determined by Etest according to the manufacturer's instructions (Sysmex bioMérieux Co., Ltd., Tokyo, Japan). Sequence analyses of the *pbp2x*, *-2b*, and *-1a* genes were performed as previously described (8, 16). Pulsed-field gel electrophoresis (PFGE) of chromosomal digests with SmaI was performed as described previously by Nagano et al. (8). The sequence type (ST) of each isolate was determined by the protocol for multilocus sequence typing (MLST) as described previously (16, 17). Phylogenetic analysis was performed on the newly identified CTB^r PSGBS isolates together with previously reported PRGBS and PSGBS clinical isolates (8, 16) and three reference strains (2603V/R, NEM316, and COH1) to explore the molecular phylogeny of *pbp* genes among the PSGBS, CTB^r PSGBS, and PRGBS isolates. The concatenated *pbp2x*, *pbp2b*, and *pbp1a* sequences were subjected to phylogenetic analyses as described previously (8, 18).

Eight clinical isolates, including six CTB^r PSGBS isolates and two PRGBS isolates, exhibited no growth inhibition zones around the ceftibuten disks. Those isolates showed increased cefaclor MICs (8 to 16 μg/ml) compared to those for the reference PSGBS isolates (Table 1). However, considerable differences were observed in ceftizoxime MIC levels among the CTB^r PSGBS, PRGBS, and reference isolates. CTB^r PSGBS and the reference isolates were all susceptible to penicillin. Nonsusceptibility to penicillin, cefotaxime, and cefepime was found in two of the PRGBS isolates tested. As shown in Table 2, two amino acid substitutions, T394A

Received 5 May 2014 Accepted 5 June 2014
Published ahead of print 11 June 2014
Editor: K. C. Carroll
Address correspondence to Yoshichika Arakawa, yarakawa@med.nagoya-u.ac.jp.

doi:10.1128/JCM.01291-14

TABLE 1 Origins and antimicrobial susceptibilities of GBS isolates

Strain or category[a] and isolate no.	Date of isolation (mo/day/yr)	Patient status[b]	Ward[c]	Age (yr)	Sex[d]	Specimen type[e]	Serotype	CTB inhibition zone diameter[f] (mm)	MIC (μg/ml) of[g]:																
									CEC	PEN	AMP	CTM	CTX	CRO	CDN	FEP	CFM	ZOX[h]	MEM	ERY	CLR	CLI	LVX	TET	VAN
2603V/R ATCC BAA-611							V	19.0	1	0.06	0.12	0.5	≤0.06	0.12	≤0.06	≤0.5	0.5	0.064	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4	0.5
NEM316 ATCC 12403							III	18.7	1	0.06	0.12	≤0.5	≤0.06	≤0.12	≤0.06	≤0.5	0.5	0.094	≤0.12	≤0.12	≤0.12	≤0.12	0.5	>4	0.5
PRGBS																									
A1	12/5/2011	Out	EM	99	M	TTA	Ib	No zone	16	0.5	0.25	>4	1	0.5	0.5	1	1	>32	0.5	>1[i]	>1[i]	>1[i]	>8	≤0.5	0.5
A2	1/12/2012	In	IM 2C	77	M	TTA	Ib	No zone	16	0.5	0.25	4	1	1	0.5	1	>1	>32	0.25	1[i]	1[i]	>1[i]	>8	≤0.5	0.5
CTB[r] PSGBS																									
B1	1/8/2012	In	IM 2C	77	M	TTA	Ib	No zone	8	0.12	0.12	2	0.25	0.25	0.12	≤0.5	1	1.5	≤0.12	≤0.12	≤0.12	≤0.12	>8	≤0.5	0.5
B2	5/16/2011	In	S 4C	80	M	Pus (oral cavity)	Ib	No zone	16	0.06	0.12	1	0.12	≤0.12	≤0.06	≤0.5	1	0.75	≤0.12	>1[j]	>1[j]	>1[j]	>8	>4	0.5
B3	9/12/2011	In	IM 2C	68	M	TTA	Ib	No zone	16	0.06	0.12	1	0.12	≤0.12	≤0.06	≤0.5	1	0.75	≤0.12	≤0.12	≤0.12	≤0.12	>8	≤0.5	0.5
B4	9/14/2011	In	NS 3B	92	F	Urine	Ib	No zone	8	0.06	0.12	0.5	0.12	≤0.12	≤0.06	≤0.5	0.5	0.75	≤0.12	≤0.12	≤0.12	≤0.12	>8	≤0.5	0.5
B5	10/18/2011	In	IM 2C	64	M	TTA	Ib	No zone	8	0.06	0.12	2	0.25	≤0.12	0.12	≤0.5	1	1.5	≤0.12	≤0.12	≤0.12	0.12	>8	≤0.5	0.5
B6	1/18/2012	In	IM 4W	94	M	TTA	Ib	No zone	8	0.06	0.12	1	0.12	≤0.12	≤0.06	≤0.5	0.5	1	≤0.12	>1[k]	>1[k]	>1[k]	>8	>4	0.5
PSGBS																									
B7	10/17/2011	Out	EM	73	M	Urine	Ib	17.6	2	0.06	0.12	1	0.12	≤0.12	≤0.06	≤0.5	1	0.19	≤0.12	≤0.12	≤0.12	≤0.12	>8	≤0.5	0.5

[a] PRGBS, GBS isolates with reduced penicillin susceptibility; CTB[r] PSGBS, PSGBS with no growth inhibition zones around a ceftibuten disk; PSGBS, penicillin-susceptible GBS.
[b] Out, outpatient; In, inpatient.
[c] S, surgery; IM, internal medicine; NS, neurosurgery; EM, emergency.
[d] M, male; F, female.
[e] TTA, transtracheal aspirate.
[f] Determined by standard disk diffusion test. CTB, ceftibuten.
[g] CEC, cefaclor; PEN, penicillin; AMP, ampicillin; CTM, cefotiam; CTX, cefotaxime; CRO, ceftriaxone; CDN, cefditoren; FEP, cefepime; CFM, cefixime; ZOX, ceftizoxime; MEM, meropenem; ERY, erythromycin; CLR, clarithromycin; CLI, clindamycin; LVX, levofloxacin; TET, tetracycline; VAN, vancomycin.
[h] MIC results of the Etest.
[i] Positive for *ermT/R*.
[j] Positive for *ermB*, *ermT/R*, and *mefA/E*.
[k] Positive for *ermB*.

TABLE 2 MLST and amino acid substitutions in PBPs among serotype Ib GBS clinical isolates

Category[a] and isolate no.	MLST sequence type	Amino acid substitution in[b]: PBP 2X					PBP 2B	PBP 1A
PRGBS								
A1	1	—[c]	A400V	V405A	—	Q557E	T567I	—
A2	1	—	A400V	V405A	—	Q557E	T567I	—
CTB^r PSGBS								
B1	1	T394A	—	—	G429S	—	T567I	—
B2	1	T394A	—	—	—	—	T567I	T145A
B3	1	T394A	—	—	G429S	—	T567I	—
B4	1	T394A	—	—	—	—	T567I	T145A
B5	1	T394A	—	—	G429S	—	T567I	—
B6	1	T394A	—	—	—	—	T567I	T145A
PSGBS								
B7	10	—	—	—	—	—	—	—

[a] PRGBS, GBS isolates with reduced penicillin susceptibility; CTB^r PSGBS, PSGBS with no growth inhibition zones around a ceftibuten disk; PSGBS, penicillin-susceptible GBS.
[b] Amino acid substitutions compared to the sequences of *Streptococcus agalactiae* strains 2603V/R and NEM316.
[c] —, no substitution was observed on the basis of the amino acid sequences of the reference strains described above.

in PBP 2X and T567I in PBP 2B, were shared among the CTB^r PSGBS isolates. A unique amino acid substitution, G429S in PBP 2X, was also observed among CTB^r PSGBS isolates B1, B3, and B5. Moreover, a T145A substitution in PBP 1A was also found among CTB^r PSGBS isolates B1, B3, and B5. The PRGBS isolates A1 and A2 shared A400V, V405A, and Q557E substitutions in PBP 2X and a T567I substitution in PBP 2B (Table 2).

PFGE patterns of the CTB^r PSGBS isolates were mutually different, although a probable close genetic relatedness was suggested between clinical isolates B1 and B5. PRGBS isolates A1 and A2, which were recovered separately from an outpatient and an inpatient, respectively, shared the same PFGE profile. Note that CTB^r PSGBS isolate B1 and PRGBS isolate A2 showed different PFGE patterns, despite being isolated from the same inpatient. CTB^r PSGBS and PRGBS isolates were assigned to ST1, a founder of clonal complex 1 (CC1) (Table 2).

In the phylogenetic analyses of concatenated *pbp* genes shown in Fig. 1, CTB^r PSGBS isolates B1 through B6 formed a clade and then formed a sister clade with four PRGBS isolates, A1 and A2 from this study and R3 and R4 from a previous report (8). All 10 of these isolates belonged to ST1. The PSGBS isolate B7 formed a clade together with other PSGBS isolates, including those from reference strain 2603V/R, which were assigned to ST10.

PRGBS isolates were reported in the United States and Canada after our first investigation of their emergence and molecular mechanisms, but they are still rare (6–11). Domestically, our subsequent investigations revealed the increase of PRGBS among clinical GBS isolates, their tendency toward resistance to multiple drugs, and a probable association of such a multidrug-resistant PRGBS clone with nosocomial spread (16). Most of those PRGBS isolates belonged to the MLST CC1, which includes the ancestral genotype ST1 (16, 19). In the present study, we report the molecular characteristics of CTB^r PSGBS. To date, disappearance of a growth inhibition zone around a ceftibuten disk or an elevation in the cefaclor MIC has not been described in penicillin-susceptible β-hemolytic streptococci. The common substitutions found in the CTB^r PSGBS isolates (T394A in PBP 2X and T567I in PBP 2B) have already been found in several PRGBS clinical isolates (6, 8, 16), but the contribution of these amino acid substitutions to β-lactam resistance remains unclear. The G429S substitution in PBP 2X was found to be a different amino acid substitution at the same position with the G429D substitution that was previously identified in PRGBS isolates (7). The T145A substitution in PBP 1A, which was the substitution on the N-terminal side of the transglycosylase domain, was not detected in previously identified PRGBS isolates, although the T145A substitution would have no effect on the ceftibuten resistance phenotype. Because the PFGE patterns of six CTB^r PSGBS isolates differed somewhat from each other, the hospital transmission of a single CTB^r PSGBS clone during a short period would be rather unlikely. The PFGE profiles of the CTB^r PSGBS isolates also differed from those of the serotype Ib PRGBS isolates A1 and A2, which were isolated during the same study period and had the apparent key substitutions V405A and Q557E in PBP 2X. Interestingly, PRGBS isolate A2 was isolated 4 days after the isolation of CTB^r PSGBS isolate B1 from transtracheal aspirate cultures from the same patient, and both isolates shared a T567I substitution in PBP 2B, but they did not have any common substitutions in PBP 2X, as shown in Table 2. These findings contradicted our initial assumption that the CTB^r PSGBS isolate might change to a PRGBS isolate after acquisition of reduced susceptibility to penicillin through accumulating mutations in its *pbp* genes. The CTB^r PSGBS and PRGBS isolates, which have emerged independently from an ancestral PSGBS strain, may have cocolonized in the respiratory tract of the host. This speculation would be supported by the findings that six CTB^r PSGBS isolates and two PRGBS isolates were all assigned to ST1, and these two groups formed sister clades with each other in the phylogenetic tree of the *pbp* genes. The EUCAST recently established clinical breakpoints for penicillin and streptococcus groups A, B, C, and G (http://www.eucast.org/clinical_breakpoints/), including a resistance criterion for penicillin at an MIC of >0.25 μg/ml. The EUCAST also notes that β-lactam susceptibility, including cefaclor and ceftibuten susceptibility in β-hemolytic streptococcus groups A, B, C, and G, is inferred from penicillin susceptibility. Cefaclor is one of the most widely used oral cephalosporins for the empirical treatment of acute upper respiratory infections in Japan, thus allowing for various bacterial species to be exposed to this drug. Cefaclor and ceftibuten are not included in the therapeutic

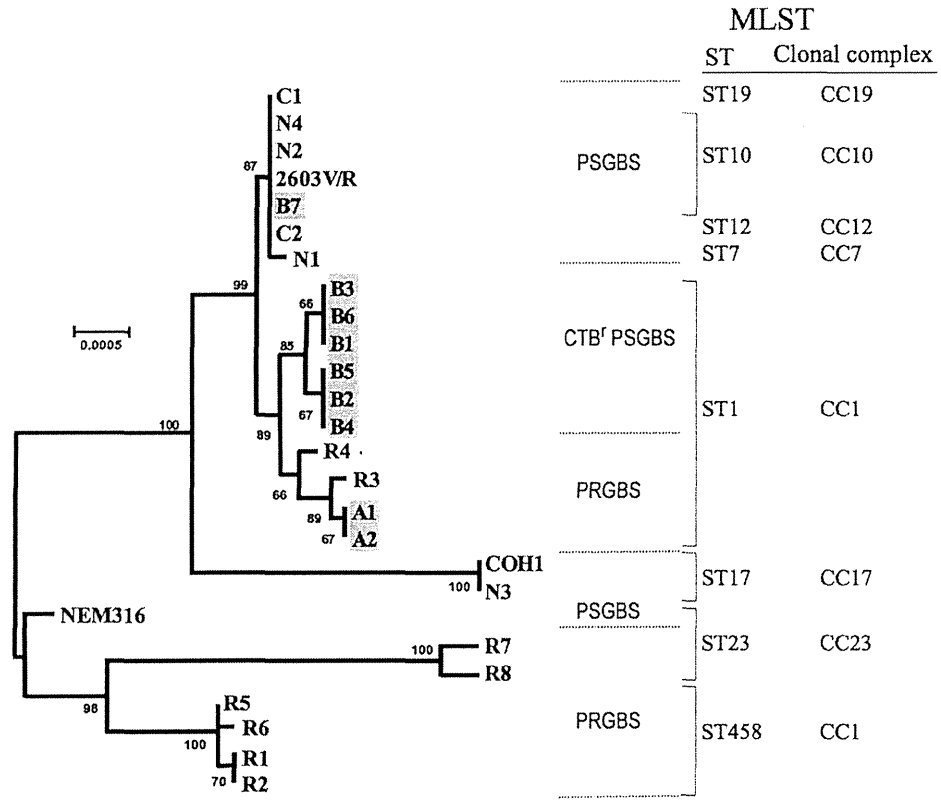

FIG 1 Correlation between phylogenetic analysis of GBS isolates based on concatenated alignment of sequenced *pbp2x*, *pbp2b*, and *pbp1a* genes and MLST sequence types. The CTB^r PSGBS isolates B1 to B6, PRGBS isolates A1 and A2, and PSGBS isolate B7, characterized in the present study, are shaded. Other isolates, such as PRGBS isolates R1 to R8 and PSGBS isolates N1 to N4, were reported previously (8). Ten PRGBS clinical isolates (strain numbers 1, 2-1, 2-2, 3, 4-1, 4-2, 5, 6, 7, and 8) caused a probable nosocomial transmission in a general hospital (16) and were identical to isolate R5 in the viewpoint of *pbp* phylogeny. Three reference strains, 2603V/R (ATCC BAA-611, GenBank accession number NC004116), NEM316 (ATCC 12403, GenBank accession number NC004368), and COH1 (GenBank accession number AAJR01000000) are included. Bootstrap support values (500 replicates) are shown as percentages. Scale bars indicate the expected number of changes per sequence position.

options for GBS infections. However, the emergence of CTB^r PSGBS clones would pose a possible problem because GBS isolates susceptible to penicillin would not necessarily be susceptible to other β-lactams, including cephalosporins, depending on the amino acid substitution(s) acquired in their PBPs.

To our knowledge, this is the first report describing the presence of a serotype Ib PSGBS lineage (i.e., CTB^r PSGBS) demonstrating unique susceptibility profiles to several cephalosporins. Lineages of CTB^r PSGBS and PRGBS, which displayed a sister clade relationship, shared an amino acid substitution (T567I) in the transpeptidase domain of PBP 2B and were assigned to ST1, suggesting a common origination. Since PBPs play an important role in bacterial viability through maintaining cellular integrity and shape, amino acid substitutions randomly acquired in PBPs would usually provide disadvantages for bacterial growth. Once a *pbp* gene mutation resulting in an amino acid substitution relevant to the resistance to β-lactams occurs in GBS, it might change to PRGBS by accumulating mutations in its PBPs, or it might evolve into CTB^r PSGBS at a low enough frequency to survive in the presence of oral cephalosporins, such as cefaclor and ceftibuten. Our findings will contribute to a better understanding of the future development of resistance to β-lactams in GBS.

Nucleotide sequence accession numbers. The *pbp* gene sequences from representative CTB^r PSGBS isolates B1 and B2 determined in this study have been deposited in GenBank under accession numbers AB819280 to AB819285.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Health, Labour and Welfare of Japan (grants H21-Shinkou-Ippan-008 and H24-Shinkou-Ippan-010) and in part by research grants for medical science from the Takeda Science Foundation (2012).

We are deeply grateful to Satowa Suzuki of the Department of Bacteriology II, National Institute of Infectious Diseases, for scientific advice in relation to epidemiology. We also thank Kyoko Osaki of Funabashi Futawa Hospital for her excellent technical assistance.

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New PCR-Based Open Reading Frame Typing Method for Easy, Rapid, and Reliable Identification of *Acinetobacter baumannii* International Epidemic Clones without Performing Multilocus Sequence Typing

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Antimicrobial resistance issues have become a global health concern. The rapid identification of multidrug-resistant microbes, which depends on microbial genomic information, is essential for overcoming growing antimicrobial resistance challenges. However, genotyping methods, such as multilocus sequence typing (MLST), for identifying international epidemic clones of *Acinetobacter baumannii* are not easily performed as routine tests in ordinary clinical laboratories. In this study, we aimed to develop a novel genotyping method that can be performed in ordinary microbiology laboratories. Several open reading frames (ORFs) specific to certain bacterial genetic lineages or species, together with their unique distribution patterns on the chromosomes showing a good correlation with the results of MLST, were selected in *A. baumannii* and other *Acinetobacter* spp. by comparing their genomic data. The distribution patterns of the ORFs were visualized by agarose gel electrophoresis after multiplex PCR amplification and digitized. *A. baumannii* sequence types (STs) corresponding to international clones I and II were successfully discriminated from other STs and *Acinetobacter* species by detecting the distribution patterns of their ORFs using the multiplex PCR developed here. Since bacterial STs can be easily expressed as digitized numeric data with plus (+) expressed as 1 and minus (−) expressed as 0, the results of the method can be easily compared with those obtained by different tests or laboratories. This PCR-based ORF typing (POT) method can easily and rapidly identify international epidemic clones of *A. baumannii* and differentiate this microbe from other *Acinetobacter* spp. Since this POT method is easy enough to be performed even in ordinary clinical laboratories, it would also contribute to daily infection control measures and surveillance.

Antimicrobial resistance has become a global health concern. The World Health Organization has stated that weak or absent antimicrobial resistance surveillance and monitoring systems accelerate the emergence and spread of antimicrobial resistance (see http://www.who.int/drugresistance/documents/surveillancereport/en/). One of the weak points of current antimicrobial resistance surveillance and monitoring systems is the absence of genetic data for the bacterial isolates. Microbial genotyping is indispensable for a precise understanding of the genetic lineages of clinical isolates that cause nosocomial outbreaks (1).

Acinetobacter baumannii is one of the major multidrug-resistant nosocomial pathogens. In particular, *A. baumannii* epidemic clones, the so-called international clones I and II, usually show multidrug resistance, and only limited antimicrobials are efficacious for treating infections caused by them (2). On the other hand, *A. baumannii* clinical isolates other than the epidemic international clones are still susceptible to several antimicrobials. The performance of appropriate precautions that target the epidemic clones is indispensable for blocking their further nosocomial transmission. Therefore, it has become very important to rapidly discriminate the *A. baumannii* epidemic clones from other nonepidemic *A. baumannii* lineages and non-*baumannii Acinetobacter* species, such as *Acinetobacter nosocomialis* and *Acinetobacter pittii*. In this regard, multilocus sequence typing (MLST) is indeed useful for the exact identification of the epidemic clones, which are classified into several sequence types (STs), such as ST1 and ST2, by MLST performed at the Institut Pasteur. ST1 and ST2 are also assigned to clonal complex 109 (CC109) and CC92, respectively, by the MLST of Bartual et al. (19) as reported by Zarrilli et al. (3) However, MLST of *Acinetobacter* clinical isolates can be performed only in limited cases of nosocomial outbreaks even in Japan, and this results in a delay in the ability to alert for the emergence and spread of epidemic clones in hospital settings. Early identification of epidemic clones of *A. baumannii* is very important especially in the areas where they have not been prevalent yet. Therefore, the establishment of easy and rapid genotyping methods has been much awaited.

The construction of new analytical methods that make it easy to obtain genetic information of clinical isolates in ordinary clinical laboratories is desired. We consider that the most convenient way to simplify microbial genotyping would be to display the re-

Received 12 April 2014 Returned for modification 6 May 2014
Accepted 28 May 2014
Published ahead of print 4 June 2014
Editor: R. Patel
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01064-14.

doi:10.1128/JCM.01064-14

TABLE 1 ORF candidates relating to small genomic islets (SGI) and their distributions among the *A. baumannii* genomes[a]

SGI ORF candidate[b]	ORF corresponding to the SGI ORF candidates among the indicated *A. baumannii* strain (Pasteur sequence type, GenBank accession no.)[c]			
	AB0057 (ST1, CP001182)	ACICU (ST2, CP000863)	ATCC17978 (ST77slv[d], CP000521)	SDF (ST17, CU468230)
ACICU_00180*		ACICU_00180 (100)	A1S_0157 (99)	
AB57_0388*	AB57_0388 (100)			
AB57_0454*	AB57_0454 (100)		Between A1S_0376[e] and A1S_0378 (99)	Between ABSDF3133 and ABSDF3134 (98)
AB57_0526	AB57_0526 (100)			Between ABSDF 3067 and ABSDF3068 (99)
ACICU_00563*		ACICU_00563 (100)		ABSDF2963 (100)
AB57_0815*#	AB57_0815 (100)		A1S_0767 (99)	
AB57_1987	AB57_1987 (100)	ACICU_01794 (99)		ABSDF1977 (98)
AB57_2085	AB57_2085 (100)		Between A1S_1754 and A1S_1755 (96)	
ACICU_01870*#		ACICU_01870 (100)	A1S_1782 (95)	ABSDF1960 (96)
ACICU_02042 *#		ACICU_02042 (100)	A1S_1927 (99)	
AB57_2484*#	AB57_2484 (100)	ACICU_02351 (98)		
ACICU_02468		ACICU_02468 (100)	A1S_2266 (99)	ABSDF1260 (97)
ACICU_02520*	Between AB57_2751 and AB57_2752 (99)	ACICU_02520 (100)	Between A1S_2318 and A1S_2319 (99)	
ACICU_02597		ACICU_02597 (100)		
AB57_2930	AB57_2930 (100)	ACICU_02697 (99)	A1S_2485 (98)	
ACICU_02886	AB57_3056 (96)	ACICU_02886 (100)	A1S_2641 (97)	
ACICU_02966*#		ACICU_02966 (100)	Between A1S_2707 and A1S_2708 (98)	ABSDF0764 (98)
AB57_3308*#	AB57_3308 (100)			
ACICU_03137*#		ACICU_03137 (100)		ABSDF0546 (98)
AB57_3624*	AB57_3624 (100)	ACICU_03369 (99)	Between A1S_3168 and A1S_3169 (99)	
ACICU_03379*		ACICU_03379 (100)		ABSDF0314 (95)
ACICU_03418*		ACICU_03418 (100)	Between A1S_3220 and A1S_3221 (99)	ABSDF0260 (100)
A1S_3257			A1S_3257 (100)	ABSDF3356 (98)
ACICU_03581*		ACICU_03581 (100)	A1S_3381 (99)	ABSDF3529 (99)

[a] SGI, small genomic islet.
[b] ORFs showing the same distribution patterns among clonal isolates are indicated by an asterisk (*), and ORFs selected for PCR-based ORF typing are indicated by a hash tag (#).
[c] Numbers in parentheses are the percent sequence similarities over representative SGI ORFs listed in the first column.
[d] slv, single locus variant.
[e] When nucleotide sequences corresponding to an SGI ORF candidate are found in the genomes of some *A. baumannii* strains but they have not been named in the annotated genome data, the ORFs flanking the nucleotide sequence similar to the SGI ORF candidate are provided.

sults as "1" for "+" and "0" for "−", the so-called binary typing, which does not require any further handling of specimens, such as performing nucleotide sequence analyses, counting the allelic repeats, or analyzing complicated restriction enzyme digestion patterns. We previously succeeded in developing a genotyping method for *Staphylococcus aureus* by detecting the distribution patterns of its open reading frames (ORFs) using multiplex PCR that can be replaced with pulsed-field gel electrophoresis (PFGE) (4, 5). In the genotyping of *S. aureus*, the distribution patterns of small genomic islets (SGIs) showed good correlations with the clonal complex (CC) types obtained by MLST. SGIs consist of one to several ORFs (6). Therefore, we hypothesized that the CCs of *A. baumannii* and the *Acinetobacter calcoaceticus-A. baumannii* complex might be also estimated or predicted by detecting the distribution patterns of SGIs specific to each *Acinetobacter* species. The distribution patterns of SGIs can easily be visualized by agarose gel electrophoresis after multiplex PCR; therefore, clone typing of isolates can be performed in many ordinary microbiology laboratories in which equipment for only PCR and agarose gel electrophoresis is available.

In the present study, therefore, we developed a new multiplex PCR-based method for easy, rapid, and reliable discrimination of the clonal complexes of *A. baumannii*, especially the epidemic clones.

MATERIALS AND METHODS

Bacterial isolates. A total of 226 *Acinetobacter* clinical isolates collected from patients in Japan between 2001 and 2012, including 79 *A. baumannii*, 20 *A. pittii*, 77 *A. nosocomialis*, 15 *Acinetobacter* species close to *A. nosocomialis*, 24 *A. radioresistens*, three *A. ursingii*, three *A. bereziniae*, two *A. soli*, one *A. junii*, one *Acinetobacter* genomic species 13BJ, and one *Acinetobacter* genomic species 14BJ, were used. These isolates were identified using their *rpoB* gene sequence (7). Two American Type Culture Collection (ATCC) reference strains available in our laboratory (*A. baumannii* strains ATCC 19606 and ATCC BAA-1605) were also used. The 79 *A. baumannii* clinical isolates and two ATCC reference strains were analyzed by MLST. The isolates were cultured overnight on soy bean casein digest agar plates at 37°C, and chromosomal DNA was extracted with the QuickGene SP kit DNA tissue (SP-DT) (Wako Pure Chemical Industries, Osaka, Japan). MLST analysis was performed according to the protocol of the Institut Pasteur MLST databases (http://www.pasteur.fr/mlst). The clustering of related STs, which was defined as a CC, was determined with the aid of the eBURST program (http://eburst.mlst.net/).

For *Acinetobacter*-specific ORF screening, *Pseudomonas aeruginosa* strain JCM 14847, *Pseudomonas putida* strain JCM 13063, *Pseudomonas fluorescens* strain JCM 5963, *Pseudomonas stutzeri* strain JCM 5965, *Pseu-*

TABLE 2 Species-specific ORF candidates

ORF[a]	Contig no., nucleotide position[b]	No. found/no. tested for *Acinetobacter* organism: *A. baumannii*	*A. pittii*	*A. nosocomialis*	*Acinetobacter* species close to *A. nosocomialis*	Other *Acinetobacter* species
pittii-1	9, 490–1095	0/8	0/4	0/8	0/4	NT[c]
pittii-2	19, 296023–297078	0/8	3/4	0/8	0/4	NT
pittii-3	21, 113290–112808	5/8	2/4	0/8	0/4	NT
pittii-4	23, 336772–337306	5/8	3/4	0/8	0/4	NT
pittii-5	23, 435468–436845	0/8	2/4	1/8	4/4	NT
pittii-6*	25, 270084–271118	0/81	19/20	0/77	0/15	0/35
pittii-7	26, 56553–57056	0/8	2/4	0/8	0/4	NT
pittii-8	31, 96908–97756	0/8	3/4	0/8	1/4	NT
pittii-9	31, 288435–286909	0/8	0/4	0/8	1/4	NT
nosocomialis-1	6, 48398–49115	0/8	0/4	7/8	0/4	NT
nosocomialis-2	12, 64890–63445	8/8	0/4	8/8	1/4	NT
nosocomialis-3*	90, 13009–11208	0/81	0/20	76/77	0/15	0/35
Asp-1*	12, 41330–40363	0/81	0/20	0/77	15/15	0/35
Asp-2	15, 52010–54341	0/8	0/4	0/8	0/4	NT
Asp-3	15, 207828–209480	0/8	0/4	0/8	0/4	NT
Asp-4	16, 155456–156670	0/8	0/4	0/8	1/4	NT
Asp-5	21, 98536–99348	0/8	0/4	0/8	0/4	NT

[a] The ORFs selected for species identification are indicated by an asterisk.
[b] ORFs were selected from *A. pittii* D499 (pittii-1 to -9 [GenBank accession no. AGFH00000000]), *A. nosocomialis* NCTC 8102 (nosocomialis-1 to -3 [GenBank accession no. AIEJ00000000]), and *Acinetobacter* species GG2 (Asp-1 to -5 [GenBank accession no. ALOW00000000]).
[c] NT, not tested.

domonas nitroreducens strain JCM 2782, *Azotobacter vinelandii* strain JCM 21475, and *Brevundimonas diminuta* strain JCM 2788 were used as a negative control. These strains were provided by the Japan Collection of Microorganisms, Riken BioResource Center (BRC), which participates in the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). *Achromobacter xylosoxidans* and *Escherichia coli* clinical isolates were also used as a negative control.

Searching small genomic islets from *A. baumannii* whole-genome sequences. The whole-genome DNA sequences of six *A. baumannii* strains, AB0057 (DDBJ/EMBL/GenBank accession no. CP001182), AB307-0294 (GenBank accession no. CP001172), AYE (GenBank accession no. CU459141), ACICU (GenBank accession no. CP000863), ATCC 17978 (GenBank accession no. CP000521), and SDF (GenBank accession no. CU468230), were obtained from an Internet database (PubMed [http://www.ncbi.nlm.nih.gov/sites/entrez]) and compared to each other using the MBGD website (http://mbgd.genome.ad.jp/) and blast+ (National Center for Biotechnology Information, Bethesda, MD, USA), with a tabular output option, and homologues were visualized by ACT (8). The nonconserved regions among the six strains were identified and selected as potential SGIs. Among the selected SGIs, those containing single to several ORFs without the presence of structures resembling insertion sequences, transposases, or integrases were selected for the determination of CCs. Nonconserved regions with larger structures, such as transposons, prophages, and antimicrobial resistance islands, were excluded. The distribution patterns of the SGI candidates (Table 1) were investigated by PCR using 42 *A. baumannii* representative clinical isolates and two ATCC strains.

Searching species-specific ORFs from whole-genome sequences. The whole-genome DNA sequences of four *A. pittii* (strains D499 [GenBank accession no. AGFH00000000], DSM 9306 [AIEF00000000], DSM 21653 [AIEK00000000], and SH024 [NZ_ADCH00000000]), two *A. nosocomialis* (strains NCTC 8102 [AIEJ00000000] and RUH2624 [NZ_ACQF00000000]), three *Acinetobacter calcoaceticus* (strains PHEA-2 [CP002177], DSM 30006 [NZ_APQI00000000], and RUH2202 [NZ_ACPK00000000]), one *A. bereziniae* (strain LMG 1003 [NZ_AIEI00000000]), one *Acinetobacter haemolyticus* (strain ATCC 19194 [NZ_ADMT00000000]), one *Acinetobacter johnsonii* (strain SH046 [NZ_ACPL00000000]), one *A. junii* (strain SH025 [NZ_ACPM00000000]), three *Acinetobacter lwoffii* (strains NCTC 5866 [AIEL00000000], SH145 [NZ_ACPN00000000], and WJ10621 [NZ_AFQY00000000]), one *Acinetobacter parvus* (strain DSM 16617 [AIEB00000000]), four *A. radioresistens* (strains DSM 6976 [AIDZ00000000], SH164 [NZ_ACPO00000000], SK82 [NZ_ACVR00000000], and WC-A-157 [ALIR00000000]), one *A. ursingii* (strain DSM 16037 [AIEA00000000]), one *Acinetobacter venetianus* (strain RAG-1 [AKIQ00000000]), and four *Acinetobacter* species that have not been given scientific names (GG2 [ALOW00000000], ATCC 27244 [ABYN00000000], HA [NZ_AJXD00000000], and NBRC 100985 [NZ_BAEB00000000]) and the genomic data of six *A. baumannii* isolates mentioned in the section above were obtained from PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and compared using blast+. ORFs showing high percent sequence similarities among all *Acinetobacter* species used in the method were selected as candidates of markers specific to *Acinetobacter* species. ORFs found only in *A. pittii*, *A. nosocomialis*, or *Acinetobacter* species close to *A. nosocomialis* (corresponding to strain GG2) genomes were selected as candidates of species-specific ORFs (Table 2). The presence of species-specific ORFs was screened by PCR for representative isolates, including eight *A. baumannii*, four *A. pittii*, eight *A. nosocomialis*, and four *Acinetobacter* species close to *A. nosocomialis*.

Multiplex PCR detection of selected ORFs to identify international clones. To maximize the discriminatory power and reliability of the identification of international clones, seven ORFs in separate SGIs were selected for multiplex PCR detection in order to identify international clones with a minimum difference of two bands in the detected ORF ladder patterns of other CCs among the isolates used in this study (Table 1).

For easy execution, the selected ORFs were detected by multiplex PCR, which we call PCR-based ORF typing (POT). The primer pairs for detecting ORFs in the seven SGIs, *Acinetobacter*-specific ORF, bla_{OXA-51} (9), and three species-specific ORFs (Table 2) were designed for multiplex PCR detection (Table 3). As ORFs in SGIs were found among *A. pittii*, *A. nosocomialis*, and *Acinetobacter* spp. close to *A. nosocomialis*, as well as for *A. baumannii*, the primers were designed to adapt universally to those *Acinetobacter* species.

Template DNAs for multiplex PCR were prepared by suspending bac-

TABLE 3 Primers finally selected for multiplex PCR

Target ORF	Primer direction	Sequence (5′ to 3′)[a]	Final concn (μM)	Amplicon size (bp)
atpA	Forward	CTGAACCTAGAACAGGATTCAGT	0.2	553
	Reverse	TCACGGAAGTATTCACCCAT	0.2	
OXA-51	Forward	GCTTCGACCTTCAAAATGCT	0.2	465
	Reverse	TCCAGTTAACCAGCCTACTTGT	0.2	
pittii-6	Forward	CATGTAGGTAGTCAAATGCCTG	0.2	401
	Reverse	CCGCTGGTGATGCTTTATTC	0.2	
nosocomialis-3	Forward	GTGATCGTGGTGATAGCTGG	0.2	362
	Reverse	GTAAGTTCCTGTTGCAACTCC	0.2	
Asp-1	Forward	GGATCTTTAACTCCATGGCTC	0.2	321
	Reverse	GATTATCrTGTAATAACCACGCAC	0.2	
AB57_2484	Forward	TATGTACAAAGCCAACCGGA	0.2	271
	Reverse	GAATTTGAGCdGAAGCCATTA	0.2	
ACICU_02042	Forward 1	CCGCGTCTTTCATAATAAGCAA	0.1	234
	Forward 2	CCACGTCTCTCATAATAAGCAA	0.1	
	Reverse 1	TGGAGAAATAGATTCTTCAAAAGTTGT	0.1	
	Reverse 2	TGCAGAAATAGATTCTTCAmAAATTGT	0.1	
ACICU_02966	Forward	ACCGTAyCCCTTTTTAATAAGTTCA	0.2	189
	Reverse	GGGCAAACTTATCATAGTTATATCGAC	0.2	
ACICU_01870	Forward	GCTGCAACCCAACCAATwA	0.2	151
	Reverse	AATTGGCTTCGhTGGATATTTATG	0.2	
AB57_3308	Forward	GCAACAGTTTCAAAATTAAATGG	0.2	122
	Reverse 1	ACTGTTTGTATGGGTATTGCAG	0.1	
	Reverse 2	ACTGTTTGTATAGGCATTGCAG	0.1	
ACICU_03137	Forward	CCyGCACTGCTCTACGATAATG	0.2	102
	Reverse	TTGyTCATAATGAAAAGCCGCA	0.2	
AB57_0815	Forward	CTTTAGAmGAGGCACGTTGGTTTG	0.2	81
	Reverse	TTTCACAyGGCTCACCGT	0.2	

[a] Mixed nucleotide residues were described according to a standard code (r, A/G; d, A/G/T; m, A/C; y, C/T; w, A/T; h, A/C/T).

terial cells in 100 μl of Tris-EDTA buffer (pH 8.0) at a turbidity of McFarland standard 0.5 to 2, heating at 100°C for 10 min, and centrifugation at 14,000 rpm (approximately 15,000 × *g*) for 1 min. Next, POT was carried out with the four thermal cyclers, i.e., GeneAmp PCR system 9700 (Life Technologies Japan, Tokyo, Japan), Applied Biosystems 2720 (Life Technologies Japan), GeneAtlas 322 (Astec, Fukuoka, Japan), and the Thermal Cycler Dice Gradient (TaKaRa Bio, Otsu, Japan), to validate their compatibility on the same platforms. The primer mixture was prepared by mixing all primers listed in Table 3 to 100× the final concentration. PCR was carried out in a 20-μl mixture containing 2 μl of the heat extract template DNA, prepared as described above, PCR buffer (3 mM Mg^{2+}), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.8 units of FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 0.2 μl of the primer mixture. The sequences and final concentrations of the primers are shown in Table 3. All four DNA preparations extracted from clinical *A. baumannii* (POT 122 [ST2] in Table 4), *A. pittii* (POT 78 in Table 4), *A. nosocomialis* (POT 105 in Table 4), and *Acinetobacter* spp. close to *A. nosocomialis* (POT 105 in Table 4) were mixed and used as the DNA template for both the positive control and the ladder marker in PCR. The thermal conditions were as follows: 95°C for 10 min, 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, and then at 4°C for several hours before agarose gel electrophoresis. The PCR products (2 μl) were electrophoresed on 4% agarose gels (NuSieve 3:1; Lonza, Basel, Switzerland) in 0.5× Tris-borate-EDTA (TBE) at 100 V for 50 min; the bands were then visualized with ethidium bromide.

After PCR, the seven SGI ORFs were scored in the order of their PCR amplicon size, with either "1" for "+"or "0" for "−" (binary code), depending on the presence or absence, respectively, of the band of amplicon DNA. These scores were then converted to decimal numbers, i.e., POT numbers. The results of each SGI binary were multiplied by 2^n ($n = 6 - 0$) and added. For example, the binary code of ST2 (1111010) was converted to 122 as follows: $1 \times 64 + 1 \times 32 + 1 \times 16 + 1 \times 8 + 0 \times 4 + 1 \times 2 + 0 \times 1$. Furthermore, each POT number was represented by a numerical label, ranging from a POT of 0 (0000000) to a POT of 127 (1111111).

RESULTS

A total of 24 SGI candidates (Table 1) were selected by comparing the whole-genome data of the six *A. baumannii* strains (AB0057, AB307-0294, AYE, ACICU, ATCC 17978, and SDF). Highly conserved (95 to 100% sequence similarity) DNA sequences were found in most of the SGIs of the six *A. baumannii* strains checked, and nucleotide sequence identities were also observed among

TABLE 4 Correlations between MLST and ORFs used for POT

POT no.	ST (CC or allele profile)	No. of isolates	Presence or absence of ORFs for the POT analysis by group:											
			ORFs for identification of bacterial species[a]					ORFs for calculation of POT no.[b]						
			atpA	OXA-51	pittii-6	nosocomialis-3	Asp-1	AB57_2484	ACICU_02042	ACICU_02966	ACICU_01870	AB57_3308	ACICU_03137	AB57_0815
A. baumannii														
122	ST2 (CC2)	27	+	+	−	−	−	+	+	+	+	−	+	−
69	ST1 (CC1)	3	+	+	−	−	−	+	−	−	−	+	−	+
0	ST235 (CC33)	1	+	+	−	−	−	−	−	−	−	−	−	−
8	ST33 (CC33)	18	+	+	−	−	−	−	−	−	+	−	−	−
8	ST148	1	+	+	−	−	−	−	−	−	+	−	−	−
32	CC33 (3-5-7-1-12-1-2)	2	+	+	−	−	−	−	+	−	−	−	−	−
32	ST239 (CC216)	1	+	+	−	−	−	−	+	−	−	−	−	−
44	New (1-4-2-1-42-1-4)	1	+	+	−	−	−	−	+	−	+	+	−	−
44	ST40	1	+	+	−	−	−	−	+	−	+	+	−	−
10	ST52	1	+	+	−	−	−	−	−	−	+	−	+	−
40	CC10 (1-3-2-1-4-1-4)	1	+	+	−	−	−	−	+	−	+	−	−	−
41	ST49	1	+	+	−	−	−	−	+	−	+	−	−	+
56	ST142	1	+	+	−	−	−	−	+	+	+	−	−	−
72	ST152	4	+	+	−	−	−	+	−	−	+	−	−	−
73	ST212	1	+	+	−	−	−	+	−	−	+	−	−	+
92	ST246	1	+	+	−	−	−	+	−	+	+	+	−	−
96	CC216 (3-4-2-2-7-2-2)	1	+	+	−	−	−	+	+	−	−	−	−	−
104	ST34 (CC34)	9	+	+	−	−	−	+	+	−	+	−	−	−
104	New (27-2v-2v-1-9-2-5)	3	+	+	−	−	−	+	+	−	+	−	−	−
104	ST145 (CC216)	1	+	+	−	−	−	+	+	−	+	−	−	−
106	CC109 (3-4-2-2-9-1-5)	1	+	+	−	−	−	+	+	−	+	−	+	−
108	ST133	1	+	+	−	−	−	+	+	−	+	+	−	−
A. pittii														
66	NA[c]	9	+	−	+	−	−	+	−	−	−	−	+	−
70	NA	1	+	−	+	−	−	+	−	−	−	+	+	−
74	NA	2	+	−	+	−	−	+	−	−	+	−	+	−
74	NA	1	+	−	−	−	−	+	−	−	+	−	+	−
76	NA	1	+	−	+	−	−	+	−	−	+	+	−	−
78	NA	6	+	−	+	−	−	+	−	−	+	+	+	−
A. nosocomialis														
97	NA	1	+	−	−	+	−	+	+	−	−	−	−	+
104	NA	26	+	−	−	+	−	+	+	−	+	−	−	−
105	NA	49	+	−	−	+	−	+	+	−	+	−	−	+
105	NA	1	+	−	−	−	−	+	+	−	+	−	−	+
Acinetobacter species close to *A. nosocomialis*														
41	NA	7	+	−	−	−	+	−	+	−	+	−	−	+
105	NA	4	+	−	−	−	+	+	+	−	+	−	−	+
109	NA	2	+	−	−	−	+	+	+	−	+	+	−	+
125	NA	2	+	−	−	−	+	+	+	+	+	+	−	+

[a] Five ORFs, i.e., *atpA*, OXA-51, pittii-6, nosocomialis-3, and Asp-1, were used in the identification of each *Acinetobacter* isolate.

[b] Seven ORFs, i.e., AB57_2484, ACICU_02042, ACICU_02966, ACICU_01870, AB57_3308, ACICU_03137, and AB57_0815, were used for calculation of the POT number of each isolate.

[c] NA, not adopted.