TABLE 2 Primers used in this study

Primer	DNA sequence (5′–3′)	Use
glpT-delta1	GCGGGATCCAGCGCGCACCGCTGTGCAG	*glpT* mutant construction
glpT-delta2	TCATGCCATTAGCCTCCGTTGCGAATACTCAACATTGAAAGCC	*glpT* mutant construction
glpT-delta3	CGGAGGCTTTCAATGTTGAGTATTCGCAACGGAGGCTAATGGC	*glpT* mutant construction
glpT-delta4	GCGGTCGACCTTCAAAGGTGTGCACCCGG	*glpT* mutant construction
torR-delta1	GCGGGATCCTCACCATGCCTGGAATATCC	*torR* mutant construction
torR-delta2	ATCAGCGGCTAAGAAATAACCTTCGTGATGTGGCATCAGAGGG	*torR* mutant construction
torR-delta3	TAAAACCCTCTGATGCCACATCACGAAGGTTATTTCTTAGCCGC	*torR* mutant construction
torR-delta4	GCGGTCGACAGCGTAACCTGTTGCAGGAG	*torR* mutant construction
torS-delta1	GCGGGATCCGCTACTACCGAGCAAAATGG	*torS* mutant construction
torS-delta2	GCTGATATTGATTCAAATCGCGTTGGTTAAATTCACGGTCGGTG	*torS* mutant construction
torS-delta3	AGTGCACCGACCGTGAATTTAACCAACGCGATTTGAATCAATATC	*torS* mutant construction
torS-delta4	GCGGTCGACTGATGACGATAAAAGCGACC	*torS* mutant construction
torT-delta1	GCGGGATCCTGGGCTGGAAATGGGCGCAG	*torT* mutant construction
torT-delta2	GATGAGGCTATCATGCGCGTGCTGTTTTATCAGCACACATCAGC	*torT* mutant construction
torT-delta3	AGCCGCTGATGTGTGCTGATAAAACAGCACGCGCATGATAGCC	*torT* mutant construction
torT-delta4	GCGGTCGACATAGCGGTGGTATCAAAACC	*torT* mutant construction
pTrc-torR-F	GCGCCATGGCACATCACATTGTTATTGTTG	pTrc99torR construction
pTrc-torR-R	GCGGGATCCTCAGCACACATCAGCGGCTAAG	pTrc99torR construction
pQE-torR-F	GCGGGATCCCCACATCACATTGTTATTG	pQE80torR construction
pQE-torR-R	GCGAAGCTTTCAGCACACATCAGCGGC	pQE80torR construction
glpT-PF	GCGGCGGCCGCTCACTTGATTGCGAGTCGCG	Probe preparation for gel shift assay
glpT-PR	GCGAAGCTTTGAAAGCCTCCGTGGCCCGTG	Probe preparation for gel shift assay
torC-PF	GCGATGCATGGGCAAGTTGATATCCAGC	Probe preparation for gel shift assay
torC-PR	GCGGGTACCAATAGCCCCTGTAAAATTATG	Probe preparation for gel shift assay
rhlR-PF	GCGGGATCCGACCAAGTCCCCGTGTCGTG	Probe preparation for gel shift assay
rhlR-PR	GCGGGATCCTCGCCATCATCCTGAGCATC	Probe preparation for gel shift assay
glpT-PR2	TGAAAGCCTCCGTGGCCCGTGGTCCTGATTCAGTGAGAGAACC	Deletion of TorR binding site in *glpT*
glpT-footprint1-6FAM	CGGCAGGTAAGCGCGCTTTG	Footprinting, 6-FAM-labeled primer
glpT-footprint2	CGCATATTCGCTCATAATTCG	Footprinting, nonlabeled primer
glpT-RACE1	GCCAGTCATTGAACCAGGCC	RACE unique primer
glpT-RACE2	GTGCACCATAGTACGACCGC	RACE unique primer
glpT-RACE3	CGCCAGAATCAAACCTGCGG	RACE unique primer
Oligo(dT)-Anchor	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV	RACE oligo(dT) attached to an anchor
PCR Anchor	GACCACGCGTATCGATGTCGAC	RACE anchor primer
rrsA-qPCR-F	CGGTGGAGCATGTGGTTTAA	Quantitative real-time PCR
rrsA-qPCR-R	GAAAACTTCCGTGGATGTCAAGA	Quantitative real-time PCR
rpoD-qPCR-F	CAAGCCGTGGTCGGAAAA	Quantitative real-time PCR
rpoD-qPCR-R	GGGCGCGATGCACTTCT	Quantitative real-time PCR
glpT-qPCR-F	TGCCCGCAGGTTTGATTC	Quantitative real-time PCR
glpT-qPCR-R	CCATGGCACAAAGCCCATA	Quantitative real-time PCR
uhpT-qPCR-F	AAGCCGACCCTGGACCTT	Quantitative real-time PCR
uhpT-qPCR-R	ACGGTTTGAACCACATTTTGC	Quantitative real-time PCR
murA-qPCR-F	CACAATTTCCGGCGCTAAA	Quantitative real-time PCR
murA-qPCR-R	GCCAGTAGAGCGGCAAAAAG	Quantitative real-time PCR
torC-qPCR-F	TTGCCGAGCGTGAATGG	Quantitative real-time PCR
torC-qPCR-R	GCGACAGGTTGCCGAGTT	Quantitative real-time PCR

dIII-digested plasmid pQE80 (Table 2). The resulting *E. coli* construct produces TorR as an N-terminal hexahistidine-tagged protein in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside).

To construct pNNglpT-P, a *lacZ* reporter plasmid used to measure *glpT* promoter activity, we PCR amplified the 300-bp region upstream of the *glpT* gene with primers glpT-PF and glpT-PR and ligated the product into NotI- and HindIII-digested plasmid pNN387 with promoterless *lacZ*. We also constructed pNNglpT-P1, which consisted of pNNglpT-P from which the TorR-binding site was deleted. A DNA fragment from which the 18-bp region containing the TorR-binding site was removed was PCR amplified with primers glpT-PF and glpT-PR2, and the following PCR was performed with primers glpT-PF and glpT-PR. The PCR fragment was ligated into pNN387 as described above. All constructs were confirmed by DNA sequencing.

Drug susceptibility assays. MIC assays were performed by a serial agar dilution method that consisted of the standard method of the Japanese Society of Chemotherapy. Bacteria were grown for 20 h at 37°C in LB medium without shaking. Five microliters of 100-fold-diluted cultures (~5,000 cells) was inoculated onto an agar plate containing antibiotics, and the plate was incubated for 16 h at 37°C. The MICs were determined to be the lowest concentration at which growth was inhibited. To examine bacterial survival rates in fosfomycin-containing broth, a 50-fold dilution of culture that had been standing overnight was inoculated into fresh LB broth, and the bacteria were grown to mid-logarithmic phase. A 1-ml portion of the cultures was transferred into a microcentrifuge tube containing fosfomycin, and the tube was incubated at 37°C without shaking for 1 h. As a fosfomycin-free control, a separate 1-ml portion of the cultures was incubated in the absence of fosfomycin. Survival

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rates are presented as the percentage of the number of CFU for fosfomycin-treated cells relative to the number of CFU for fosfomycin-free control cells.

RNA extraction and quantitative real-time PCR analyses. Bacteria were grown to mid-logarithmic growth phase (optical density at 600 nm [OD_{600}], ~0.5) in LB medium. TorR was overexpressed in the wild-type parent harboring pTrc99torR grown with 0.1 mM IPTG. TMAO was added to the LB medium at a concentration of 20 mM. Total RNA extraction and cDNA synthesis were performed using an SV total RNA isolation system and a GoScript reverse transcription system as described by the manufacturer (Promega Corp., Madison, WI). The real-time PCR mixture included 2.5 ng cDNA and 200 nM primers in SYBR Select master mix (Applied Biosystems, Foster City, CA), and PCRs were run on an ABI Prism 7900HT Fast real-time PCR system. Constitutively expressed *rrsA* and *rpoD* genes were used as an internal control. Primers are listed in Table 2. Amplification plot and melting curve data are available upon request.

Overexpression and purification of His_6-TorR. His_6-TorR was expressed in and purified from *Escherichia coli* Rosetta(DE3) (Novagen/EMD Bioscience, Philadelphia, PA). Bacteria containing recombinant plasmid were grown to an OD_{600} of 0.4 at 37°C in LB medium, 0.5 mM IPTG was then added, and culture growth was continued for 3 h. Cells were harvested and stored at −80°C overnight. The cell pellet was suspended in lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 10% glycerol) and lysed by sonication. The lysate was centrifuged, and the resulting supernatant was mixed with Ni-nitrilotriacetic acid–agarose (Qiagen, Valencia, CA) for 1 h. The agarose was washed twice with 50 mM imidazole, and then His_6-TorR was eluted with 500 mM imidazole. The protein was >95% pure, as estimated by SDS-PAGE and Coomassie brilliant blue staining. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were stored at −80°C.

Gel shift assays. To assess TorR binding to the *glpT* promoter sequence in gel shift assays, we used a 321-bp DNA probe containing the 300-bp region upstream of the *glpT* start codon. We also used a 318-bp DNA fragment from the 300-bp region upstream of the *torC* start codon as a positive-control probe and a 323-bp DNA fragment from the *Pseudomonas aeruginosa rhlR* gene as a nonspecific control probe. The purified His_6-TorR was phosphorylated in gel shift assay buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 10% glycerol) containing 50 mM carbamoyl phosphate (Sigma-Aldrich, St. Louis, MO) and 10 mM magnesium chloride for 30 min at 30°C. Then, we expected that at least some portion of the TorR protein pool would be phosphorylated in the *in vitro* reaction. The probe DNA fragments (0.30 pmol) were mixed with phosphorylated His_6-TorR in a 10-μl reaction mixture. After incubation for 20 min at room temperature, samples were separated by electrophoresis on a 5% nondenaturing acrylamide Tris-glycine-EDTA (10 mM Tris [pH 8.0], 380 mM glycine, 1 mM EDTA) gel in Tris-glycine-EDTA buffer at 4°C. The gel was incubated in 10,000-fold-diluted SYBR green I nucleic acid stain (Lonza, Walkersville, MD), and the DNA was visualized under UV light at 300 nm.

DNase I footprinting. DNase I footprinting was performed using a previously described nonradiochemical capillary electrophoresis method on an ABI Prism genetic analyzer equipped with ABI Prism GeneScan analysis software (33). The 6-carboxyfluorescein (6-FAM)-labeled (5′) 255-bp DNA fragment (starting at 49 bp inside the *glpT*-coding region and ending 206 bp upstream of the *glpT* start codon) was generated by PCR amplification using a 6-FAM-labeled forward primer (primer glpT-footprint1-6FAM) and an unlabeled reverse primer (primer glpT-footprint2). The DNA fragment (0.45 pmol) was mixed with phosphorylated TorR (40 pmol) in a 50-μl reaction mixture containing the same buffer described above. After incubation for 20 min at room temperature, DNase I (0.3 U; Promega Corp., Madison, WI) was added. After incubation for 60 s at room temperature, samples were purified for GeneScan sequencing analysis.

Analysis by 5′ RACE. We used 1.5 μg of total RNA isolated from the mid-logarithmic-phase wild-type parent grown in LB medium and synthesized cDNA using a GoScript reverse transcription system with the unique primer glpT-RACE1 (starting at 559 bp inside the *glpT*-coding region). A poly(A) linker was attached to the 3′ terminus of cDNA using the terminal transferase (TdT; New England BioLabs, Ipswich, MA). Two rounds of PCR were performed by using an oligo(dT) primer attached to a 22-base anchor sequence at the 5′ end [primer Oligo(dT)-Anchor] and primer glpT-RACE2 (starting at 441 bp inside the *glpT*-coding region) for the first round and an anchor primer without the oligo(dT) sequence (PCR anchor) and primer glpT-RACE3 (starting at 309 bp inside the *glpT*-coding region) for the second round. Eventually, we got a single ~380-bp product by rapid amplification of cDNA ends (RACE). The product was TA cloned into pCR2.1 TOPO (Life technologies, Waltham, MA), and six clones were sequenced.

Promoter assays. To measure the promoter activity of the *glpT* genes, EHEC strains carrying the reporter plasmid were grown in LB medium. The wild-type parent and a Δ*torR* strain harboring the reporter plasmid were anaerobically grown to the early stationary phase. Each cell culture of 0.5 ml was transferred into a 1.5-ml microtube, and 50 μl of chloroform was added. After incubation for 5 min, the β-galactosidase activity in the supernatant was monitored using a Tropix Galacto-Light Plus kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Fosfomycin active transport assays. Assays to test for fosfomycin accumulation in bacterial cells were conducted as previously described (21). Bacteria were grown to late logarithmic phase in 20 ml of LB medium and resuspended in 1 ml of LB medium. This suspension was incubated for 60 min at 37°C in the presence of 2 mg of fosfomycin per ml and then washed three times with hypertonic buffer (10 mM Tris [pH 7.3], 0.5 mM $MgCl_2$, 150 mM NaCl) to remove the antibiotic. Cells were resuspended in 0.5 ml of distilled water and plated on LB agar to determine the number of CFU/ml. The bacterial resuspension was boiled at 100°C for 3 min to release the fosfomycin. After centrifugation, the antibiotic concentration in the supernatant was determined by a diffusion disc assay. In this assay, sterilized assay discs (diameter, 13 mm; Whatman, Florham Park, NJ) were saturated with 0.1 ml of the supernatant and deposited onto LB agar plates overlaid with a 1:10 dilution of an overnight culture of *E. coli* MG1655 as a reporter strain (28). Commercial fosfomycin was used as a standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The fosfomycin concentration in the supernatants was quantified by the diameter (mm) of the inhibitory rings on the LB agar culture and is presented as the number of ng per 10^7 cells.

RESULTS

Overexpression of the *torR* gene confers fosfomycin tolerance in EHEC. In our previous study, we found that the activation of CpxAR resulted in increased tolerance to fosfomycin (21). We tested other TCSs of EHEC to determine if they control fosfomycin tolerance. Overexpression of the response regulator can artificially lead to constitutive activation of the TCS even in the absence of a signal from the sensor protein (31, 33, 34). We overexpressed each response regulator from an IPTG-inducible promoter on pTrc99A, a multicopy plasmid, and then the MICs of fosfomycin for these response regulator-overexpressing strains were determined. We found that the *torR*-overexpressing strain showed a lower susceptibility to fosfomycin than the control strain carrying the empty plasmid (MICs, 4 mg/liter for the control strain versus 16 mg/liter for the *torR*-overexpressing strain) (Table 3). We also compared the survival rate of the *torR*-overexpressing strain with that of the control EHEC strain after treatment with 1.56 mg/liter of fosfomycin, as described in Materials and Methods. After incubation in the presence of fosfomycin, the number of CFU of the control strain was only 1.4% ± 0.3% of the number of CFU of the control strain in the absence of fosfomycin

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TABLE 3 Fosfomycin MICs for the EHEC O157:H7 strain and its derivatives

Strain	Fosfomycin MIC (mg/liter)
Parent (HH-H7-008)	4
HH-H7-008/pTrc99A	4
HH-H7-008/pTrc99torR	16
HH-H7-008 Δ*glpT* (HH-H7-095)	16
HH-H7-008 Δ*glpT*/pTrc99A	16
HH-H7-008 Δ*glpT*/pTrc99torR	16
HH-H7-008 Δ*cpxAR*/pTrc99A	4
HH-H7-008 Δ*cpxAR*/pTrc99torR	16
HH-H7-008 Δ*cpxA* (HH-H7-040)	16
HH-H7-008 Δ*cpxA* Δ*torR* (HH-H7-157)	16

(Fig. 1). On the other hand, the survival rate of the *torR*-overexpressing EHEC strain was 88.8% ± 11.9% after fosfomycin treatment (Fig. 1). This finding is consistent with the findings of the MIC experiments and indicates that overexpression of *torR* increases the tolerance of EHEC O157:H7 to fosfomycin.

Overexpression of the *torR* gene suppresses *glpT* expression and results in reduced fosfomycin uptake. GlpT and UhpT are transporters for fosfomycin uptake, and MurA is a target for the drug; thus, they are determinants for susceptibility to fosfomycin. To determine whether TorR affects the level of *glpT*, *uhpT*, and/or *murA* gene expression or not, we compared the levels of these transcripts between the *torR*-overexpressing and control strains by quantitative PCR (qPCR) analysis. We found that the level of *glpT* expression in the *torR*-overexpressing cells was 15-fold lower than that in the control, whereas no significant difference in the levels of *uhpT* and *murA* expression was observed between these strains (Fig. 2).

To confirm that the depletion of GlpT reduces the susceptibility to fosfomycin, we constructed a mutant with an in-frame deletion of the *glpT* gene and tested the susceptibility to fosfomycin. The *glpT* deletion mutant had increased tolerance to fosfomycin compared to that of the parent strain. However, overexpression of TorR in the *glpT* mutant had no effect on fosfomycin tolerance relative to that of the strain carrying the vector control (Table 3). These observations suggest that fosfomycin tolerance in the *torR*-overexpressing strain is attributed to the suppression of GlpT production. We previously found that the Cpx system represses *glpT* expression. The response regulator CpxR was constitutively activated when CpxA phosphatase activity was abolished, and then fosfomycin tolerance was increased (21). Our data suggest that there is no cross talk between the Cpx and Tor pathways because the overexpression of TorR in the *cpxAR* mutant and the deletion of *cpxA* in the *torR* mutant still increased fosfomycin tolerance to the same degree as that in the wild-type parent background (Table 3).

We also measured the intracellular fosfomycin levels of the *torR*-overexpressing and control strains. The level of fosfomycin accumulation in the *torR*-overexpressing cells was 7.5-fold lower than that in cells of the control strain (7.0 ± 0.8 ng per 10^7 cells for the *torR*-overexpressing strain versus 52.6 ± 19.4 ng per 10^7 cells for the control strain) (Fig. 3). From these combined results, the induction of fosfomycin tolerance by *torR* overexpression can be

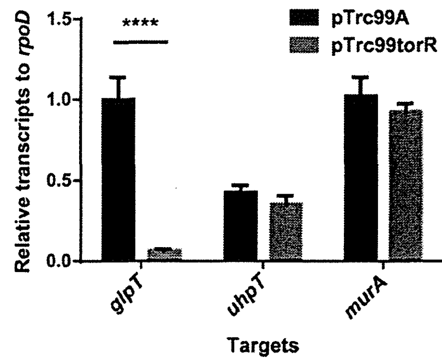

FIG 2 Levels of *glpT*, *uhpT*, and *murA* gene transcripts in the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid). The levels of the *glpT*, *uhpT*, and *murA* transcripts are presented relative to the *rpoD* gene (a housekeeping gene) transcript level. Data are plotted as the means from two biological replicates; error bars indicate ranges. ****, $P < 0.0005$.

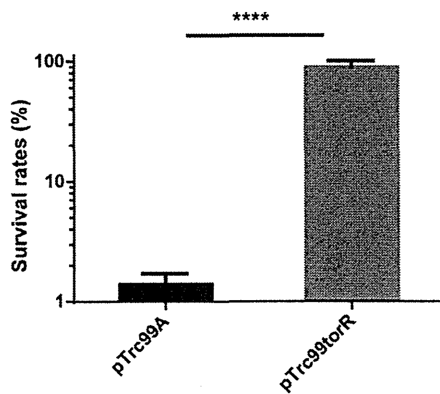

FIG 1 Survival rates of the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) after incubation with or without 1.56 μg/ml fosfomycin. The survival rates are presented as the percentage of the number of CFU/ml for the strain after incubation with fosfomycin relative to that after incubation without fosfomycin. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

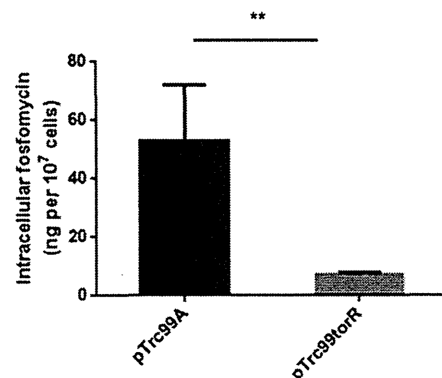

FIG 3 Intracellular accumulation of fosfomycin in the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid). Accumulation is described as the amount of fosfomycin (ng) in 10^7 cells. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. **, $P < 0.01$.

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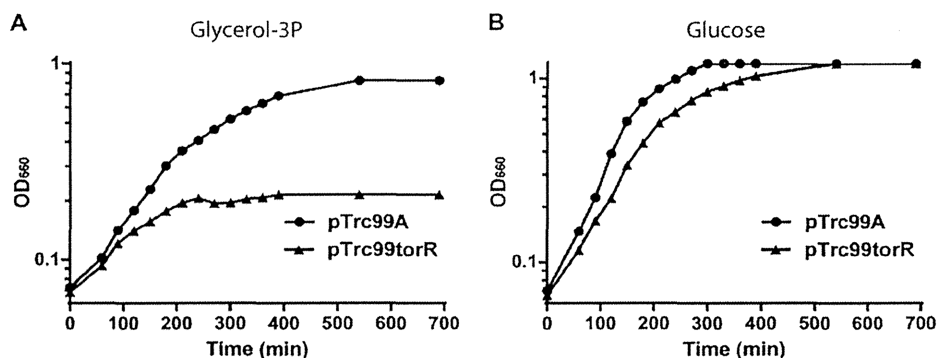

FIG 4 Cell growth of the wild-type parent strain harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) in DMEM with 0.5% (wt/vol) glycerol-3-phosphate (glycerol-3P) (A) or glucose (B) as a carbon source to support growth. Growth was monitored by determination of the absorbance at 660 nm. The experiment was repeated twice, and similar results were obtained each time.

explained by the reduction of GlpT-dependent fosfomycin uptake.

TorR-overexpressing cells have limited glycerol-3-phosphate uptake. GlpT transports glycerol-3-phosphate into the cells as its innate function, and then EHEC can utilize it as a carbon source. We predicted that the *torR*-overexpressing strain has a growth defect in a medium containing glycerol-3-phosphate as the carbon source to support growth. We separately cultured EHEC carrying a *torR*-overexpressing plasmid or an empty vector in glucose-free DMEM supplemented with glycerol-3-phosphate as the carbon source to support growth. As shown in Fig. 4A, the growth rate of the *torR*-overexpressing strain was lower than that of the control strain in this medium. However, there was no significant difference in growth between these strains when they were grown in DMEM supplemented with glucose, in which growth does not depend on GlpT, instead of glycerol-3-phosphate (Fig. 4B).

TMAO represses *glpT* expression in a TorR-, TorS-, and TorT-dependent manner. Trimethylamine-*N*-oxide (TMAO) is a signal that activates the Tor pathway in EHEC anaerobiosis. TorT, a periplasmic receptor protein, forms a complex with TMAO. TorS is autophosphorylated by interaction with the TorT-TMAO complex and then transfers its phosphate to TorR (23–25). To test whether TMAO represses *glpT* expression through activation of the Tor pathway, we measured the levels of the *glpT* transcript in the wild-type EHEC and *torR* mutant strains grown anaerobically in the presence or absence of TMAO by qPCR analysis. The qPCR data showed that the level of expression of *glpT* in the wild-type parent was 2.5-fold lower when it was grown with TMAO than when it was grown without TMAO (Fig. 5A). On the other hand, the level of the *glpT* transcript in the *torR* mutant was not decreased by TMAO (Fig. 5A). We also confirmed that the level of expression of *torC*, which is activated by the Tor system, was increased in the wild-type parent grown in the presence of TMAO but not in the *torR* mutant (Fig. 5A). Similar to the results for the *torR* mutant, neither repression of *glpT* nor activation of *torC* by TMAO was observed in the *torS* and *torT* mutant strains because TorS and TorT are also required for Tor activation in the presence of TMAO (see Fig. S1 in the supplemental material). Consistent with the results of qPCR analysis, fosfomycin accumulation in EHEC grown with TMAO was 2.2-fold lower than that in EHEC grown without TMAO (472 ± 117 ng per 10^7 cells when the strain was grown without TMAO versus 213 ± 83 ng per 10^7 cells when the strain was grown with TMAO) (Fig. 5B). These results indicate that TMAO activates the Tor pathway and then suppresses GlpT production.

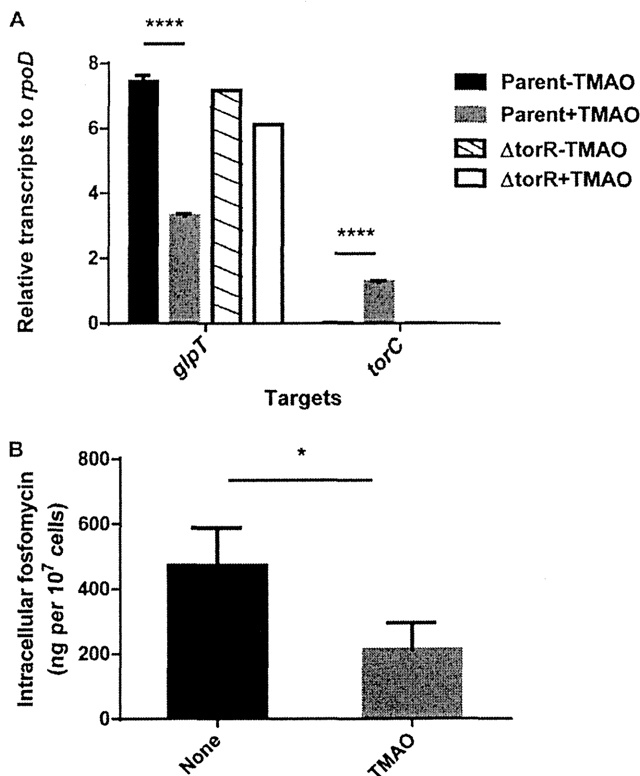

FIG 5 Effect of TMAO-induced Tor system activation. (A) The levels of the *glpT* and *torC* transcripts in the wild-type parent and the *torR* mutant grown anaerobically with or without 20 mM TMAO were measured by qPCR. These transcript levels are presented relative to the *rpoD* gene (a housekeeping gene) transcript level. Data are plotted as the means from two biological replicates; error bars indicate ranges. (B) Intracellular accumulation of fosfomycin, described as the amount of fosfomycin (ng) in 10^7 cells, in the wild-type parent anaerobically grown with or without 20 mM TMAO. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. *, $P < 0.05$; ****, $P < 0.0005$.

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FIG 6 TorR binds the *glpT* promoter region containing predicted decameric sequence. (A) Results of a gel shift assay showing the binding of TorR to the *glpT* promoter. The TorR protein (0, 10, 20, or 40 pmol) was added to reaction mixtures containing 0.3 pmol of a DNA probe. DNA upstream of *torC* and *rhlR* was used as a positive control and a nonbinding (negative) control, respectively. Reaction mixtures were separated on polyacrylamide gels. Free and TorR-bound DNAs were visualized by SYBR green I staining under UV light at 300 nm. (B) DNase I footprinting of the *glpT* promoter region. A 255-bp, 6-FAM-labeled DNA fragment was incubated in the presence or absence of phosphorylated TorR (40 pmol) and subjected to DNase I digestion. The fluorescence intensities of the DNA fragments (*y* axis) are plotted relative to their size in bases (*x* axis). One region (outlined by the dashed line) was protected from DNase I digestion in the presence of 40 pmol TorR. (C) Sequence of the upstream *glpT* region. The double-headed arrows indicate the predicted TorR-binding sites, and the region protected from DNase I digestion is indicated by the box. The transcriptional start site is indicated by the arrow labeled +1. The *glpT* translational start codon and predicted −10 and −35 boxes are indicated.

TorR is a repressor for the *glpT* gene. To investigate if TorR directly regulates the expression of *glpT*, we purified TorR as an N-terminal hexahistidine-tagged protein and examined its ability to bind the promoter of the *glpT* gene by gel shift assays. We used a 321-bp DNA probe including a 300-bp region upstream of the *glpT* gene. We also used a similarly sized DNA probe from the region of the *torC* promoter including a known TorR-binding sequence as a positive control and a similarly sized DNA probe from the region upstream of the *rhlR* gene from *P. aeruginosa* as a nonspecific control. TorR bound to both *glpT* and *torC* promoter DNA fragments but not the *rhlR* gene used as a nonspecific probe (Fig. 6A). We also confirmed that the binding to the *glpT* promoter depends on the phosphorylation of the TorR protein because no shift of the *glpT* DNA band was observed when nonphosphorylated protein (in the absence of carbamoyl phosphate) was used.

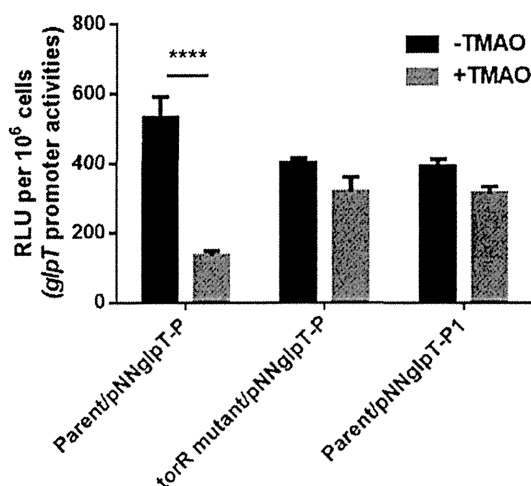

FIG 7 β-Galactosidase activities of the EHEC wild-type parent and the *torR* mutant containing *glpT-lacZ* reporter plasmids anaerobically grown with or without 20 mM TMAO. The β-galactosidase activities from LacZ expression in these strains correspond to *glpT* promoter activities and are described as the number of relative light units (RLU) in 10^6 cells. Data are plotted as the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

TorR is proposed to bind to a decameric consensus sequence (5′-CTGTTCATAT-3′) (35). The promoter region of the *torC* gene has three consensus sequences that match completely and another one with a 3-base replacement to which the TorR protein binds with a relatively lower affinity (35). We found these consensus sequences with the 3-base replacement 41 bp upstream (CTGTTAATCA) (designated site 1), and 119 bp upstream (CAATTCACAT) (designated site 2) of the *glpT* translational start site, respectively (the consensus sequences are underlined). We then identified the TorR-binding region by DNase I footprinting. A 33-bp region including the site 1 element was partly protected from DNase I digestion by TorR, suggesting that the TorR protein binds to site 1 but not to site 2 (Fig. 6B). The *glpT* transcriptional start site has been proposed to be an A residue 76 bases downstream of the translational start site; the site 1 element is located 36 bases upstream of the transcriptional start site (36). We confirmed that the proposed transcriptional start site is correct by 5′ RACE analysis, as described in Materials and Methods (Fig. 6C).

To verify the TorR-binding site in an *in vivo* experiment, we constructed two *glpT*-promoter-*lacZ* plasmids; one was designated pNNglpT-P, which contains a 300-bp region upstream of the *glpT* translational start site fused to a promoterless *lacZ* gene, and the other was designated pNNglpT-P1, which contains the proposed TorR-binding site on pNNglpT-P from which an 18-bp region was removed. The LacZ activity in the wild-type parent containing plasmid pNNglpT-P was decreased when the strain was anaerobically grown with TMAO, but this decrease did not occur in the *torR* mutant (Fig. 7). In addition, TMAO did not repress LacZ expression in the wild-type parent carrying pNNglpT-P1 (Fig. 7). These observations indicate that TorR binds the decameric consensus sequence (CTGTTAATCA, where the underlined sequence is the consensus sequence) between the transcriptional and translational start sites of *glpT* and then represses *glpT* gene transcription probably by blocking transcription elongation.

Fosfomycin does not activate the Tor pathway. In our previous study, we showed that fosfomycin activates the Cpx pathway and induces tolerance to fosfomycin by repressing the expression of *glpT* (21). To investigate whether or not the Tor pathway is also activated by fosfomycin, we grew the wild-type parent in medium with and without 0.2 μg/ml of fosfomycin and compared the levels of the *torC* transcript between these strains by qPCR analysis. Cell growth was not affected with this concentration of fosfomycin. No significant difference in *torC* transcript levels between the strains was seen when they were grown with and without fosfomycin (see Fig. S2 in the supplemental material).

Overexpression of *torR* orthologs in the nonpathogenic K-12 strain and a UPEC strain also induces tolerance to fosfomycin. To determine if the Tor system induces fosfomycin tolerance in other *E. coli* species, we used strain MG1655 from the nonpathogenic K-12 subgroup and nongastrointestinal infectious uropathogenic *E. coli* (UPEC) strain CFT073. The sequences of the open reading frames for the *torR* and *glpT* orthologs among the MG1655, EHEC O157, and UPEC CFT073 strains were >95% identical (28, 29). We cloned *torR* ortholog genes of MG1655 and CFT073 into plasmid pTrc99A and then overexpressed the plasmid in parallel hosts. Similar to the results of the experiment with EHEC O157, survival rates after fosfomycin treatment were higher for the *torR*-overexpressing strains than for the vector control (9.1% ± 2.3% for the MG1655 control strain versus 100.7% ± 4.3% for *torR*-overexpressing strain MG1655 and 7.4% ± 2.1% for the CFT073 control strain versus 45.3% ± 5.7% for *torR*-overexpressing strain CFT073) (Fig. 8).

DISCUSSION

The decline in the use of fosfomycin, as well as the biological cost conferred by the acquisition of resistance to fosfomycin, has contributed to a lower incidence of fosfomycin resistance. In an era of a shortage of new antimicrobial agents, particular interest in fosfomycin has recently resurfaced. We aimed to get insight into the molecular mechanism by which innate tolerance is induced; such insight will aid us with the establishment of a method that enhances the efficacy of this drug.

Reversible mechanisms to control fosfomycin tolerance that do not depend on genetic modifications, including chromosomal mutations in *glpT*, *uhpT*, and/or *murA*, would allow EHEC to relieve the biological cost conferred by fosfomycin resistance in fosfomycin-free circumstances and could thus be beneficial for EHEC in respect to fitness.

We previously identified a reversible mechanism operated by the CpxAR two-component system (21). Activation of the Cpx pathway leads to increased tolerance to fosfomycin and decreased uptake of the carbon substrates glycerol-3-phosphate and glucose-6-phosphate due to repression of GlpT and UhpT production when a sub-MIC of fosfomycin is present.

In this study, we found that the Tor pathway induces fosfomycin tolerance (Table 3; Fig. 1). Activation of the Tor pathway reduced the level of *glpT* expression (Fig. 2, 4, and 5A) and decreased the amount of intracellular fosfomycin, which led to increased tolerance to fosfomycin (Fig. 3 and 5B). However, unlike the Cpx pathway, the Tor pathway was not activated by fosfomycin (see Fig. S2 in the supplemental material) and TorR directly impaired *glpT* transcription elongation, resulting in a decrement of GlpT protein production without cross talk with the Cpx pathway (Table 3; Fig. 6). We also found that the Tor orthologs in a nonpatho-

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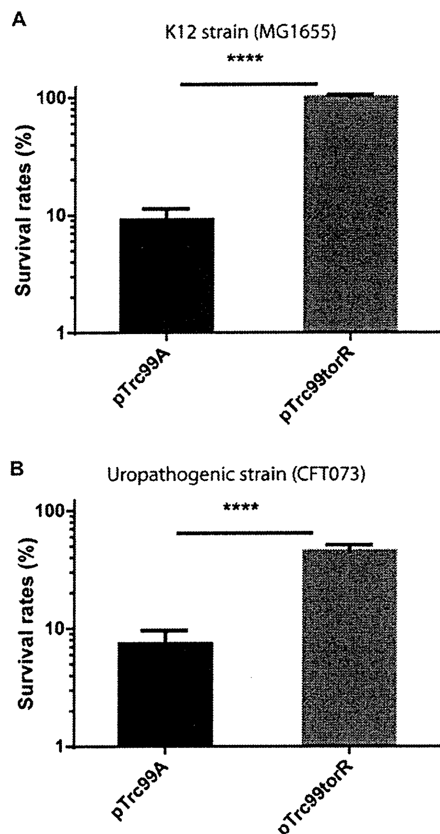

FIG 8 Survival rates of nonpathogenic K-12 (MG1655) (A) and uropathogenic (CFT073) (B) strains harboring pTrc99A (vector control) or pTrc99torR (a TorR-overexpressing plasmid) after incubation with or without 1.56 μg/ml fosfomycin. The survival rates are described as the percentage of the number of CFU/ml for the strain after incubation with fosfomycin relative to that after incubation without fosfomycin. Data plotted are the means from three independent experiments; error bars indicate standard deviations. ****, $P < 0.0005$.

genic K-12 strain and a nondiarrheic uropathogenic strain (UPEC) also participate in the induction of fosfomycin tolerance (Fig. 8). The Tor-mediated fosfomycin tolerance mechanism may be conserved among strains of *E. coli* and related species.

The Tor pathway was originally characterized to be a regulatory system for the anaerobic reduction of TMAO (35). *E. coli* species, including EHEC, use TMAO as an electron acceptor for their anaerobic respiration, where TMAO is reduced to trimethylamine (TMA) to allow the cell to generate the proton motive force required for ATP synthesis (37). The Tor pathway is activated when TMAO is present in the medium. TorS is autophosphorylated when the complex of TMAO and TorT interacts with the sensing domain of this protein and then transfers the phosphate to its cognate response regulator, TorR (24). Phosphorylated TorR stimulates the promoter of the *torCAD* operon, encoding proteins that are responsible for TMAO respiratory system (38). The Tor pathway also activates the *tnaA* gene, encoding tryptophanase, which is proposed to counteract alkaline stress, and represses the *gadAB* and the *hdeABD* genes, which contribute to bacterial survival under acidic pH conditions (26). Regulation by Tor also contributes to protection against alkalization of the medium after TMA production. In addition to these genes, we have found another gene regulated by the Tor system.

The TMA precursor is produced by gut microorganisms from carnitine, lecithin, or phosphatidylcholine contained in food (for example, red meat) and oxidized into TMAO in liver, and eventually, the oxidized molecule is transported via blood to the enteric site (39). Therefore, the activation of the Tor pathway by TMAO could occur when EHEC is in the animal gut. Animal studies have indicated that TMAO levels can be affected by the gut microflora, which produces TMA from dietary carnitine and phosphatidylcholine, resulting in an increased risk for cardiovascular disease (CVD) (40, 41). These results imply that the activity of the Tor system may be associated with the levels of TMAO derived from the TMA producers in the gut, which could affect the level of susceptibility to fosfomycin in *E. coli* species.

E. coli species can use glycerol-3-phosphate as an electron donor for anaerobic respiration, where it is oxidized to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase and transfers electrons to the terminal reductases, including the TMAO reductase (37, 42). Thus, repression of GlpT production by Tor activation may lead to defects in not only carbon source availability but also anaerobic electron transport. However, if any alternative carbon sources and electron donors are present at sufficient levels in the gut, cells with fosfomycin tolerance conferred by transient Tor activation may be able to grow without being outcompeted by the susceptible members.

Genetic modifications, such as chromosomal mutations and acquisition of external genes, can provide bacteria with long-term drug resistance that leaves genetic scars and then often confers an irreversible fitness burden. We suggest that the mechanism of control of tolerance to antibiotics that depends on environmental changes that do not leave any genetic scars, which is often difficult to detect in clinical situations by current molecular techniques, may be an important clinical issue to keep in mind. However, this theory encourages us to create a concept that we can make fosfomycin treatment more effective if we can close reversible mechanisms, such as the Cpx and Tor pathways.

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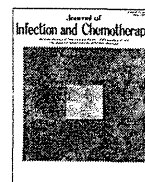

Original article

Loop-mediated isothermal amplification assay for 16S rRNA methylase genes in Gram-negative bacteria

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ABSTRACT

Using the loop-mediated isothermal amplification (LAMP) method, we developed a rapid assay for detection of 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*), and investigated 16S rRNA methylase-producing strains among clinical isolates. Primer Explorer V3 software was used to design the LAMP primers. LAMP primers were prepared for each gene, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). Detection was performed with the Loopamp DNA amplification kit. For all three genes (*rmtA*, *rmtB*, and *armA*), 10^2 copies/tube could be detected with a reaction time of 60 min. When nine bacterial species (65 strains saved in National Institute of Infectious Diseases) were tested, which had been confirmed to possess *rmtA*, *rmtB*, or *armA* by PCR and DNA sequencing, the genes were detected correctly in these bacteria with no false negative or false positive results. Among 8447 clinical isolates isolated at 36 medical institutions, the LAMP method was conducted for 191 strains that were resistant to aminoglycosides based on the results of antimicrobial susceptibility tests. Eight strains were found to produce 16S rRNA methylase (0.09%), with *rmtB* being identified in three strains (0.06%) of 4929 isolates of *Enterobacteriaceae*, *rmtA* in three strains (0.10%) of 3284 isolates of *Pseudomonas aeruginosa*, and *armA* in two strains (0.85%) of 234 isolates of *Acinetobacter* spp. At present, the incidence of strains possessing 16S rRNA methylase genes is very low in Japan. However, when Gram-negative bacteria showing high resistance to aminoglycosides are isolated by clinical laboratories, it seems very important to investigate the status of 16S rRNA methylase gene-harboring bacilli and monitor their trends among Japanese clinical settings.

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1. Introduction

Some Gram-negative bacilli are highly resistant to aminoglycosides (AGs), including strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Serratia marcescens*. In Japan, such resistant strains were first reported in 2003 [1]. These AG-resistant bacteria acquire genes similar to the 16S rRNA methylase genes of actinomyces, which confer resistance to clinically used AGs antibiotics such as gentamicin and amikacin.

So far, at least eleven 16S rRNA methylase genes (*rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, *armA*, and *npmA*) have been identified [1–8], and since these genes are usually present on plasmids, they can easily transfer to other bacteria. When multiple drug-resistant *P. aeruginosa* acquires a 16S rRNA methylase gene, antibiotic therapy would become ineffective. Accordingly, the emergence and spread of such bacteria should be carefully monitored, and investigating the acquisition of 16S rRNA methylase genes by clinical isolates is important for both prevention and treatment of their infections, so development of a rapid and convenient detection method would be desirable.

Detection of AGs-resistant strains can be done by antimicrobial susceptibility testing with methods such as broth microdilution method in accordance with the CLSI's protocol, but such procedures

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Table 1
LAMP primers (F3, B3, FIP, BIP, LF, and LB) for the target 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) were designed by Primer Explorer Ver.3 software (Eiken Chemical Co., Ltd. Tokyo, Japan).

16S rRNA methylase	Amplified region (GenBank accession number)	Primer	Type	Sequence (5′–3′)
rmtA	7118–7328 (AB120321)	rmtA-F3	F3	GGATTGGACTTCACGTTCGC
		rmtA-B3	B3c	TTGCTTCCATGCCCTTGC
		rmtA-FIP	F2–F1c	CTCGCTCCAGCAAAGGCAGTAATGCAGGATGTGATGTGTACG
		rmtA-BIP	B1–B2c	GGCGCTACTGCAGGCACTAGTAAACTGCGGGTGGGGAA
		rmtA-LB	LB	TACCCCTCGGATTGCCGT
rmtB	1776–2001 (AB103506)	rmtB-F3	F3	GGCATTGCATCCGTGTGG
		rmtB-B3	B3c	GCGGGGTATTGAGGGATTG
		rmt-FIP	F2–F1c	CACATCCTGCAGGGCAAAGGTAAGGGATTGGGGGATGTCAT
		rmt-BIP	B1–B2c	GGCGACCTGGCGCTGATTTTAAGTGCCATGGCAGAACC
		rmtB-LF	LFc	CCAATCTTTTTCCCTAGCAAAGGG
		rmtB-LB	LB	TTTTGCCCCTGCTGGAGCG
armA	2281–2539 (AB116388)	armA-F3	F3	GGAAATATCAAACATGTCTCATCT
		armA-B3	B3c	GCTGTTTTAGCACAGGAAG
		armA-FIP	F2–F1c	CTCAGCTCTATCAATATCGTATGCATGGCTTCAATCCATTAGCTTTA
		armA-BIP	B1–B2c	AGCATTATTGGGAAGTTAAAGACGAACATCATAAGTACCTTTGTAGAC
		armA-LF	LFc	TTTTTCATTTTCATTCCATTGGT
		armA-LB	LB	AGGTTTTTGAATAAAGAGAGTGA

are time-consuming because bacterial culture in broth media containing some AGs is required after identification of bacterial species. In addition, the presence or absence of 16S rRNA methylase genes cannot be determined by the routine microbiology laboratory testing. Although tests based on the polymerase chain reaction (PCR) have been developed for detection of resistance genes, PCR requires special equipment and is unsuitable for an ordinary hospital laboratory. In contrast to PCR, loop-mediated isothermal amplification (LAMP) is an easy and rapid method for genetic testing [9,10].

Accordingly, we designed LAMP primers to detect three 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) that have been reported to be associated with AG resistance in Japan. Then we employed the LAMP method to investigate the frequency of 16S rRNA methylase gene-positive isolates among the clinical isolates identified in clinical microbiology laboratories around Japan.

2. Materials and methods

2.1. Design of primers and LAMP assay method

For specific detection of three 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*), a set of LAMP primes (two outer primers; F3 and B3, two inner primers; FIP and BIP, and two loop primers; LF and LB) was designed for each gene using Primer Explorer Ver.3 software (Eiken Chemical Co., Ltd. Tokyo, Japan). The GenBank accession numbers of *rmtA*, *rmtB*, and *armA* which were used for the design are AB120321, AB103506, and AB116388, respectively.

The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd.). The reaction was performed in a volume of 25 μl containing 40 pmol each of the FIP and BIP primers, 5 pmol each of the F3 and B3 primers, 20 pmol each of the LF and LB primers, 2× reaction mixture (12.5 μl), *Bst*-DNA polymerase (1 μl), and template DNA (2 μl). For *rmtA*, an LF primer was not used because it could not be designed by the Primer Explorer Ver.3. Extraction of template DNA was conducted by incubating bacterial strains at 95 °C for 10 min and 2 μL of the supernatant was obtained by 10,000 rpm and 1 min of centrifugation. The reaction was performed at 65 °C for 60 min, and the amplified products in the reaction tube were detected by visual inspection using Loopamp fluorescent detection reagent (Eiken Chemical Co., Ltd.).

Primers were synthesized by a contract laboratory (Nihon Gene Research Laboratory Inc., Miyagi, Japan).

3. Evaluation of LAMP assay performance

The detection sensitivity and reaction time were investigated by using serial dilutions of PCR products for *rmtA*, *rmtB*, and *armA*. For real-time assay performance, a real-time turbidimeter (Loopamp LA-320C, Eiken Chemical Co., Ltd.) was used. The specificity of the LAMP assay was investigated by using nine bacterial species (65 strains) (Table 2) obtained from the National Institute of Infectious Diseases, which were verified to possess 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) by PCR and DNA sequencing.

4. Investigation of clinical isolates

At 33 medical institutions throughout Japan (3 in Hokkaido, 2 in Tohoku, 14 in Kanto, 4 in Chubu, 2 in Kinki, 3 in Chugoku, 4 in Shikoku, and 1 in Kyushu), agreement of the institutional head was obtained to investigate a total of 5998 strains isolated from January through December 2008, consisting of 3056 *Enterobacteriaceae* strains, 2885 *P. aeruginosa* strains, and 57 *Acinetobacter* spp. strains. From among these isolates, 132 strains with resistance to AGs (gentamicin and/or amikacin) were identified, including 52 *Enterobacteriaceae* strains, 77 *P. aeruginosa* strains, and 3 *Acinetobacter* spp. strains. 132 strains of AGs resistance were measured by the LAMP method.

Table 2
Specificity of the LAMP assay for bacterial strains confirmed to possess *rmtA*, *rmtB*, or *armA*.

16S rRNA methylase[a]	Strain	*n*	*rmtA*-LAMP	*rmtB*-LAMP	*armA*-LAMP
rmtA	*P. aeruginosa*	21	21	–	–
rmtB	*C. freundii*	1	–	1	–
	E. coli	7	–	7	–
	K. pneumoniae	5	–	5	–
	S. marcescens	1	–	1	–
armA	*Acinetobacter* sp.	3	–	–	3
	A. baumannii	4	–	–	4
	C. freundii	1	–	–	1
	E. aerogenes	1	–	–	1
	E. cloacae	3	–	–	3
	E. coli	3	–	–	3
	K. pneumoniae	10	–	–	10
	S. marcescens	5	–	–	5

[a] 16S rRNA methylase genes were verified by PCR and DNA sequencing.

In addition, another three institutions (Tohoku University Hospital, Yamagata University Hospital, and Miroku Medical Laboratory Co., Ltd. (Nagano, Japan)) provided a total of 2449 strains that were isolated from August 2013 through May 2014, consisting of 1873 *Enterobacteriaceae* strains, 399 *P. aeruginosa* strains, and 177 *Acinetobacter* spp. strains. Among these isolates, 59 strains with resistance to AGs (gentamicin and/or amikacin) were identified, consisting of 41 *Enterobacteriaceae* strains, 11 *P. aeruginosa* strains, and 7 *Acinetobacter* spp. strains. 59 strains of AGs resistance were measured by the LAMP method.

Evaluation of antimicrobial susceptibility was performed in accordance with CLSI M100 S-18, and resistance was defined as an MIC $\geq$16 μ/mL for gentamicin and an MIC $\geq$64 μ/mL for amikacin [11].

Information about the bacterial strains investigated in this study was limited to bacterial species name of each isolate and the results of antimicrobial susceptibility testing, with no clinical data being collected.

5. Results

5.1. LAMP primers

The sequences of the LAMP primers for the 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) are shown in Table 1. For *rmtA*, there was only a single loop primer (LB).

5.2. Assay performance

When the detection sensitivity of the LAMP assay was investigated, it was found that *rmtA*, *rmtB*, and *armA* could be detected at 10^2 copies/tube (Fig. 1).

The reaction time was 15 min for 1.0×10^7 copies/tube and 32 min for 1.0×10^2 copies/tube in the case of *rmtA*, while the respective times were 23 min and 52 min for *rmtB*, and 27 min and 41 min for *armA*.

The specificity of the assay was investigated using 9 species (65 strains) of bacteria known to possess 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) and the results are shown in Table 2. The LAMP assay for *rmtA* detected all of the strains possessing the *rmtA* gene (1 species, 21 strains), while strains with *rmtB* or *armA* were not detected. Similarly, the assays for *rmtB* and *armA* specifically detected strains possessing *rmtB* (4 species, 14 strains) and strains containing *armA* (8 species, 30 strains), respectively, with no false positive or false negative results.

5.3. Clinical isolates

The clinical isolates from 2008 and 2013–2014 included a total of 191 AG-resistant strains. Among the *Enterobacteriaceae* isolates, 3 strains were positive for *rmtB* (1 strain each of *Escherichia coli*, *Enterobacter cloacae*, and *Citrobacter freundii*). In addition, 3 strains of *P. aeruginosa* were positive for *rmtA* and 2 strains of *Acinetobacter* spp. were positive for *armA*. The 16S rRNA methylase gene detection rate was 0.06% for *Enterobacteriaceae*, 0.10% for *P. aeruginosa*, and 0.85% for *Acinetobacter* spp. (Table 3).

6. Discussion

Concerning 16S rRNA methylase-producing strains, *rmtA* was first discovered from *P. aeruginosa* in Japan as a gene for the enzyme that causes methylation of 16S rRNA at 1405G [1]. Thereafter, *armA*, *rmtB*, *rmtC*, *npmA*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, etc. have been identified [2–4,12–14]. Wachino and Arakawa has been reported that $bla_{CTX-M-type}$, $bla_{OXA-type}$, $bla_{TEM-type}$, $bla_{SHV-type}$, $bla_{NDM-type}$, or $bla_{KPC-type}$ positive isolate harbors 16S rRNA methylase encoding gene [15]. Such strains have been found among *K. pneumoniae*, *S. marcescens*, *P. aeruginosa*, *C. freundii*, and *Acinetobacter baumannii* [2–4,12–14]. Moreover, *Salmonella enterica* producing ArmA has been reported in Bulgaria, the USA, and the UK, while *Shigella flexneri* producing ArmA was reported in Bulgaria [15].

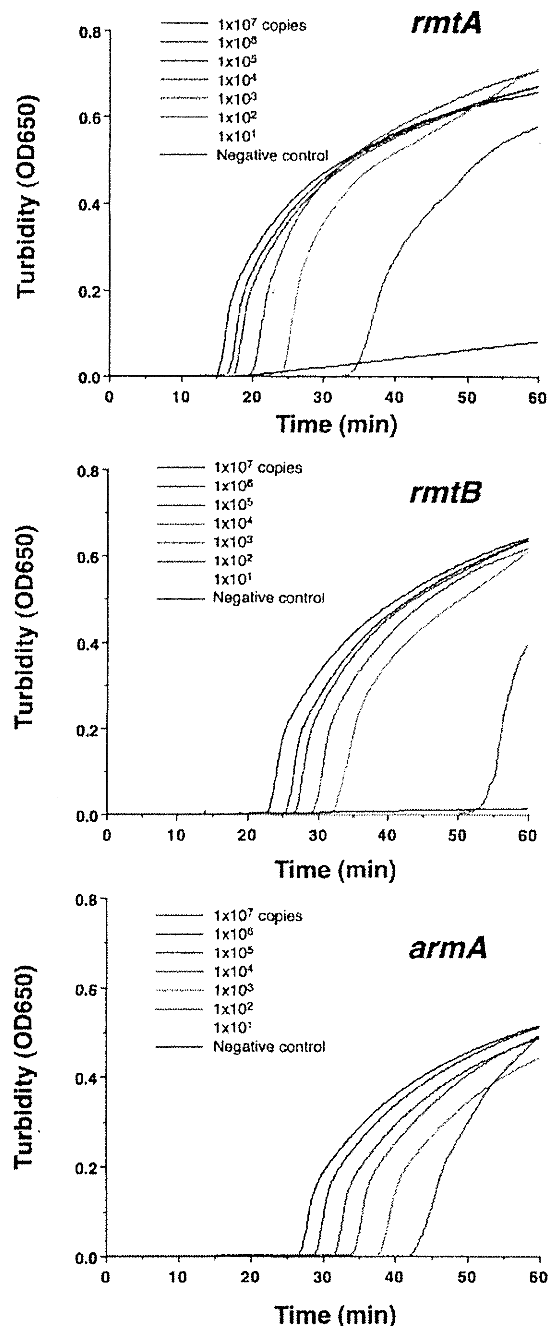

Fig. 1. Detection sensitivity and reaction time of the LAMP assays for *rmtA*, *rmtB*, and *armA*. Turbidity was monitored by a Loopamp real-time turbidimeter LA-320 at 650 nm.

In the present study, LAMP primers were designed to detect three 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) that have been reported to be associated with resistance in Japan. To reduce the reaction time, a loop primer (LF, LB) was also used [16].

Table 3
Detection of strains possessing *rmtA*, *rmtB*, or *armA* among 191 aminoglycoside-resistant strains identified from 8447 clinical isolates.

Bacterial species	Isolates	Resistance to AMK or GM		Strains with 16S rRNA methylase genes, *n*			% Of strains producing 16S rRNA methylase
		n	%	*rmtA*	*rmtB*	*armA*	
Enterobacteriaceae	4929	93[a]	1.89	0	3[b]	0	0.06
P. aeruginosa	3284	88	2.68	3	0	0	0.10
Acinetobacter sp.	234	10	4.27	0	0	2	0.85
Total	8447	191	2.26	3	3	2	0.09

AMK, amikacin; GM, gentamicin.
[a] *E. coli* (70 isoletes), *P. mirabilis* (6), *K. pneumonia* (6), *E. cloacae* (5), *P. stuartii* (2), *C. freundii* (1), *K. oxytoca* (1), *S. marsescens* (1), *S. plymuthica* (1).
[b] *E. coli* (1 isolete), *E. cloacae* (1), *P. stuartii* (1).

Assessment of the sensitivity of this assay showed that it could detect the target genes at 10^2 copies/tube in less than 60 min. In the future, detection of 16S rRNA methylase may be able to be directly carried out from clinical samples, such as a blood culture bottle. With regard to specificity, the LAMP assay for *rmtA*, *rmtB* and *armA* detected all strains, with no false positive or false negative results. Accordingly, this LAMP assay is easy to perform, can detect target genes for AG resistance within 60 min, and demonstrates high specificity, suggesting that it may be useful for clinical detection and surveillance of strains with high AG resistance. The LAMP method can be employed as an infection control test in medical institutions where PCR equipment is not available and it seems important to utilize this method as a rapid test for detecting 16S rRNA methylase producing strains.

The only previous surveillance of 16S rRNA methylase-producing strains was conducted by Yamane et al., in 2004 [17]. They investigated 87,626 Gram-negative clinical isolates for 16S rRNA methylase genes and identified *rmtB*, *armA* in 0.02% of *E. coli*, *rmtA* in 0.08% of *P. aeruginosa*, and *armA* in 0.13% of *Acinetobacter* spp. In the present investigation, 16S rRNA methylase genes were detected with *rmtB* being found in 0.06% of *Enterobacteriaceae*, *rmtA* in 0.10% of *P. aeruginosa*, and *armA* in 0.85% of *Acinetobacter* spp. These results were comparable to those reported by Yamane et al. in terms of the bacterial species, genes, and detection rates.

Currently, the prevalence of strains possessing 16S rRNA methylase genes is very low in Japan and there is no trend for a marked increase. However, it has been shown in other countries that the transmissible plasmid carrying the NDM-1 gene also carries the *armA*, *rmtB*, or *rmtC* genes. When Gram-negative bacteria showing high resistance to amikacin or gentamicin are isolated by clinical laboratories, it seems worth considering to perform the LAMP assay method to rapidly identify the 16S rRNA methylase gene-harboring Gram-negative bacilli and monitor their trends among Japanese clinical settings.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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耐性病原体 up-to-date～耐性メカニズムから治療戦略まで～

III　耐性菌サーベイランス

2．感染対策サーベイランスにおける新しい取り組み
－耐性菌時代の院内感染対策と 2DCM-web －

New era of surveillance － 2DCM-web and Infection control of the MDRO era －

藤本 修平*

菌の院内拡散は抗菌薬による選択圧とともに，耐性菌による院内感染拡大の主たる加速要因である。院内感染症の起因菌は常在菌や環境菌であり，検出されること自体は異常と言えず，さらに，菌は目に見えないため，異常な検出であったとしてもその広がりを把握することが困難である。筆者らは菌の院内拡散を可視化する方法の研究を行ってきた。平成21年(2009年)より菌の院内拡散状況を可視化する2DCM(Two-dimensional color-coded Carrier Mapping system)を厚生労働省院内感染対策サーベイランス(JANIS)検査部門参加施設が自由に利用できるようにする研究を行った。平成23年(2011年)度より2DCM-webとして利用できるようになり，すでに400を超える施設が利用している。これまで全国サーベイランスの還元情報は，印刷物，電子媒体で表やグラフの形で行われており，2DCM-webのようなアプリケーションを用いてデータを高次利用する情報還元は海外でも行われていない。さらに現在，菌の不自然な分離を自動検出して菌の院内拡散を感度よく発見する方法や，それを利用して院内感染対策の問題点の指摘，対策の評価を行う方法などについても将来的に全国サーベイランスで利用可能になるように検討を進めている。本稿では耐性菌時代の院内感染対策と菌の院内拡散について述べた後，菌の院内拡散を発見，解析する方法，2DCM，2DCM-webの実際について説明する。

Key Words：感染対策／耐性菌／菌の院内拡散／2DCM-web

I　今日の院内感染対策の問題点と対策

1．高度先進医療と易感染患者の増加

医療の現場では医療の高度先進化にともない侵入門戸での生体防御を妨げるさまざまな医療行為が行われており，易感染患者を生んでいる。易感染患者と言うと抗がん剤などによるいわゆる免疫が低下した患者を思い浮か

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べることが多いが，多くの日和見感染は皮膚粘膜での防御を障害された患者から発生する。皮膚の物理的な防御を妨げる血管内カテーテルによるカテーテル関連血流感染，気道の防御機構である繊毛運動や粘液分泌細胞の働きを気管内挿管によってバイパスすることによる人工呼吸器関連肺炎等，デバイス関連感染症が問題となるのはそのような背景による。

2．易感染患者と日和見感染症

病原体は排泄門戸から感染経路を介して侵入門戸に達した後，付着，侵入，細胞内侵入，毒素生産などの病原性によって感染症を発症させる。我々の体はこれらの病原性に対して侵入門戸での防御を含むさまざまな生体防御能により守られている。健康な個体のすべての生体防御能を破って感染症を起こす病原体を「病原菌」あるいは「強毒菌」と呼んでいる。インフルエンザウイルスや腸管出血性大腸菌は健康な若者に感染症を起こす「病原菌」，「強毒菌」の例である。

一方，侵入門戸での防御など生体防御が妨げられると「病原菌」，「強毒菌」以外の菌（非病原菌，弱毒菌）によっても感染症が発生する。正常な皮膚や粘膜を破って体の中に入ることができる病原体は少ないが，たとえば，一旦血管内に入ってしまえば感染症を起こす菌は多い。このような，非病原菌，弱毒菌が起こす感染症を日和見感染症と呼び，その原因となる菌を日和見感染菌と呼んでいる。

3．高度先進医療と多剤耐性菌による日和見感染症

高度先進医療の実施によって易感染患者が生まれ，易感染患者は非病原菌による日和見感染症を発症する。我々の身の回りにいる非病原菌とは，常在細菌，環境菌であり，これらの菌が日和見感染症の原因となる。常在菌や環境菌は，患者，医療従事者，病院環境の一部として長時間，病院内に存在する。病院内には易感染患者が居るため治療・予防目的で抗菌薬が多用される。常在菌や環境菌は病院内に長時間存在するため，くり返し抗菌薬に曝露され，感性菌は淘汰され，耐性菌，くり返しの曝露により多剤耐性菌が選択されるようになる。常在菌や環境菌には免疫による排除が起こらないため，これらの菌では治療・予防目的で抗菌薬が使用されるたびに，ごくわずかな耐性菌でも確実に選択される。さらに，高度先進医療では一時的であっても患者の動きが制限されることが多く，医療従事者が，治療のため，ケアのために患者に触れる必要があり，院内感染の機会を多くしている。高度先進医療が耐性菌による難治院内感染症を生むのは必然と言える。

4．耐性菌時代の感染対策

高度先進医療は易感染患者を作るため安全な実施のために有効な抗菌薬が必要であるが，今日，抗菌薬の開発が低調でこの基盤が揺らいでいる。

対策として耐性菌による院内感染を抑制する方法について考えてきた。耐性菌による院内感染対策には科学的データに基づいて，①院内感染を抑止すること，②耐性菌の拡散を抑止することが必要で，耐性菌の拡散の抑止のためには抗菌薬の適正使用による選択圧の除去が重要である。科学的データを作るためのサーベイランスシステムの構築から着手し[1]，院内感染対策の評価，抗菌薬適正使用の評価に結びつくようなデータの収集，集計，還元法を考えてきた[2]。

5．耐性菌時代の院内感染対策の問題点

日和見感染菌による院内感染対策には，①日和見感染菌は常在菌や環境菌であって分離されても異常とは言えないこと，②菌は目に見えないため広がりが把握できないという問題があり，院内感染対策の担当者は病原菌（強毒菌）による感染症や検出されることがまれな耐性菌による感染以外では院内感染であることを自信をもって判定できないという問題がある。

II 耐性菌時代の感染対策と菌の院内拡散

院内感染症には，感染の原因となる病原体が直接個体の外から付着・侵入して発症する外因性院内感染症と，感染源がその個体の常在細菌叢である内因性院内感染症がある。外因性院内感染症では何らかの感染経路によって環境あるいは別の個体から病原体が患者に付着することが必要で，菌の院内拡散が最初のステップとなる。耐性菌の院内拡散においても菌の院内拡散が最初の重要なステップとなるが，少量の感染が発生しても選択圧がない状態では耐性菌は脱落することが多いため，実際の拡散にはさらに抗菌薬による選択圧も重要な役割を果たす。内因性院内感染症においても耐性菌の院内拡散によって患者が入院後に他の患者から多剤耐性菌など難治化の原因となる菌をもらい，抗菌薬による選択圧によって常在細菌叢がその耐性菌で置き換わった場合，難治化の原因は菌の院内拡散と抗菌薬による選択圧にあると言える。さらに，ひとりの患者から他の患者に菌が拡散する状況は衛生的でない状態を意味し，アウトブレイクの危険因子となる（図1）。耐性菌時代の院内感染対策では菌の院内拡散の抑制と抗菌薬の適正使用が重要である。

III 菌の院内拡散の検出と見える化

本稿I-5で述べたように，菌の院内拡散を抑制するためには菌の院内拡散を異常として検出し，さらに見える化することが必要である。筆者らは，常在菌，環境菌を含めたあらゆる菌について，菌の不自然な分離（異常集積）を自動的に検出することで菌の院内拡散を早期に発見する方法（「菌の異常集積の自動検出」〔PMA：Probability-based Microbial Alert〕），PMAの出す警告をスコア化し

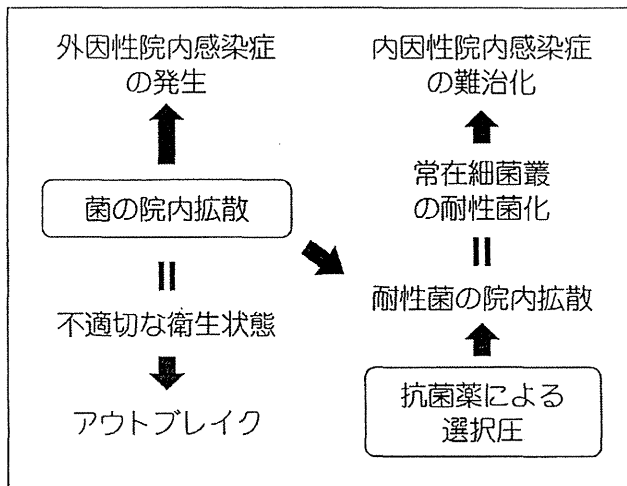

図1 耐性菌時代の院内感染症と菌の院内拡散，耐性菌による選択圧
菌の院内拡散は外因性院内感染症の最初のステップであり，耐性菌の院内拡散においても重要なステップである。さらに，内因性院内感染症難治化の原因，アウトブレイクの危険因子でもある。
（筆者作成）

PMA（Probability-based Microbial Alert；菌の異常集積の自動検出）

て院内感染対策の問題点を予測し，また，院内感染対策の評価を支援する指標となる「菌の異常集積警告スコア累積」（Σ-alert），「菌の異常集積スコア累積マトリクス」（Σ-alert matrix）および個々の菌の院内拡散を見える化する 2DCM（Two-dimensional color-coded Carrier Mapping system）を開発した。これらを概説する。

1．「菌の異常集積の自動検出」（PMA）

個々の菌種について，何もないときにその菌が分離される人の頻度であるベースラインレート（baseline rate），一定期間に検出の対象となった人数（検体提出患者数），そのうちその菌が分離された人数（陽性患者数）の３つがわかると，そのような菌の分離がまったく偶然だけに支配されて（sporadic に）発生する確率は二項分布によって正確に求めることができる（図2）。したがって，菌の分離がまったく偶然だけに支配されていることを帰無仮説とすると，求めた確率が一定以下であれば，「偶然だけに支配されて起こった確率は低いため，人為的関与があったと考えた方が自然である」という解釈ができる（帰無仮説が否定される）。人為的関与とは，ある個体から別の個体に菌を広げる，何らかの感染源があってそこから菌が広がるなどを意味する。同時に，「自然に分離されるよりも，異常に多い人数から菌が分離された」こと，つまり時間的，あるいは空間的な集積を意味する。これは菌の院内拡散と同等と考えてよい。

$$p_{m\geq k}=\sum_{m=k}^{n} {}_nC_m p^m(1-p)^{n-m}$$

図2　二項分布による確率の計算

n：検査をした人数，m：その菌が検出された人の数，p：その菌が分離される人の率（ベースラインレート）

二項分布は分布という名前がついているが，結果としての確率分布を示す用語で，確率の計算は母集団の分布に影響されない。菌の分離が偶然だけに左右されて発生していることを帰無仮説として計算された確率が小さいとき，この帰無仮説は否定され，人為的な力の影響があったと判断する。これは偶然だけに左右されている sporadic な分離でなく，異常な集積（菌の院内拡散）があったことを意味する。

（筆者作成）

PMA は数理統計学の統計的仮説検定の基本を応用したものであるが，異常の検出を実用的に行うためには，毎日，検出される全菌種について，病院全体，病棟別などの場所（ユニット）ごとに，さらに，短期間に起きる問題，長期に起こる問題の両方を確実にとらえるために複数の集計期間で計算を行う必要がある。すなわち，菌種数×ユニット数×集計期間（通常７日，14 日，28 日または 30 日）の計算を毎日行うことが必要となり，人手で行うことは困難であるが，データの収集，集計，確率の計算を自動的に行うシステムを構築すれば漏れのない異常の検出を行える[3]。

PMA は，最初に国立大学共通ソフトウエア感染症管理システム（プロジェクト終了）[4]，次に SHIPL（Standardized Hospital Infection Primary Lookout system）[5][6]に実装され，利用されている。

2．「菌の異常集積警告スコア累積」（Σ-alert），「菌の異常集積スコア累積マトリクス」（Σ-alert matrix）

PMA は求めた確率をもとに，偶然以外に左右されて発生した可能性の高さによって level 1 ～ 3 までの警告を出す。レベルの基準値（しきい値）は任意に設定可能であるが，現在稼働しているシステムの既存値は，level 1：$p < 0.01$，level 2：$p < 0.005$，level 3：$p < 0.001$ である。

2DCM（Two-dimensional color-coded Carrier Mapping system）
SHIPL（Standardized Hospital Infection Primary Lookout system）

PMAの院内感染予防に関する有用性を検討する中で，level 1～3の警告をそのまま，1～3の値として警告が出るたびに加算し，月ごとに集計，さらに棒グラフ化するΣ-alertの仕組み（Σ-alert）を検討した。Σ-alert（図3）を用いることで数年分の菌の異常集積（院内拡散の状況）を理解することができる[3)]。

Σ-alertでは統計的に有意な変化だけを積算するため，菌の分離があっても統計的に有意な異常でなければスコアは0になる。

Σ-alertは1枚のグラフに数年間分の1菌種の菌の異常集積（院内拡散の状況）を示すことができる。縦軸の累積警告スコアの値をカラーコード化（色分けで表す方法）すると1枚のグラフを1本の帯として表すことができる。これを縦軸方向に重ねると数十菌種の数年分の菌の異常集積を1枚の図にすることができる（図4）。これを警告スコア累積カラーマトリクスΣ-alert matrixと呼んでいる。

Σ-alert matrixは個々の施設の感染対策上の問題点を見出す目的で開発を行ったが，全分離菌種についての数年間の経過をひと目で見られることから，院内感染対策上のわずかな変化もよく反映することが明らかになった（図4，5）。

Σ-alertはSHIPLに実装され利用されているが，Σ-alert matrixの実装は今後の課題である。PMA，Σ-alert，Σ-alert matrixの算出，作図に必要な情報は厚生労働省院内感染対策サーベイランス（JANIS）検査部門で収集されている情報であり，これらの仕組みをJANIS提出データを利用した処理に用いることも原理的には可能であるが，PMAの算

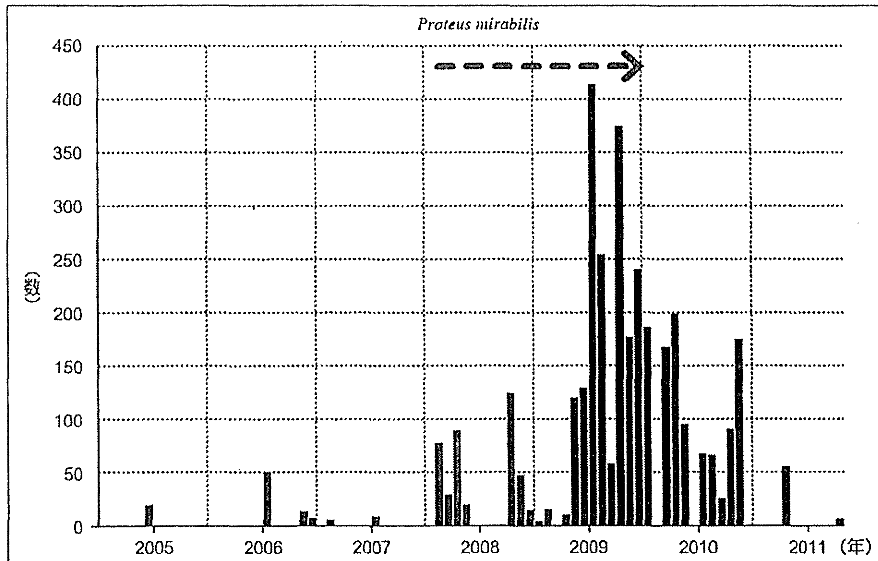

図3　Σ-alert

「菌の異常集積の自動検出」（PMA：Probability-based Microbial Alert）の例。本稿図7の2DCMの例と同じ施設の*Proteus mirabilis*のもの。点線矢印の期間が，本稿図7で示した患者Aから*Proteus mirabilis*がくり返し分離されるようになった時期を示している。

（筆者作成）

JANIS（院内感染対策サーベイランス）

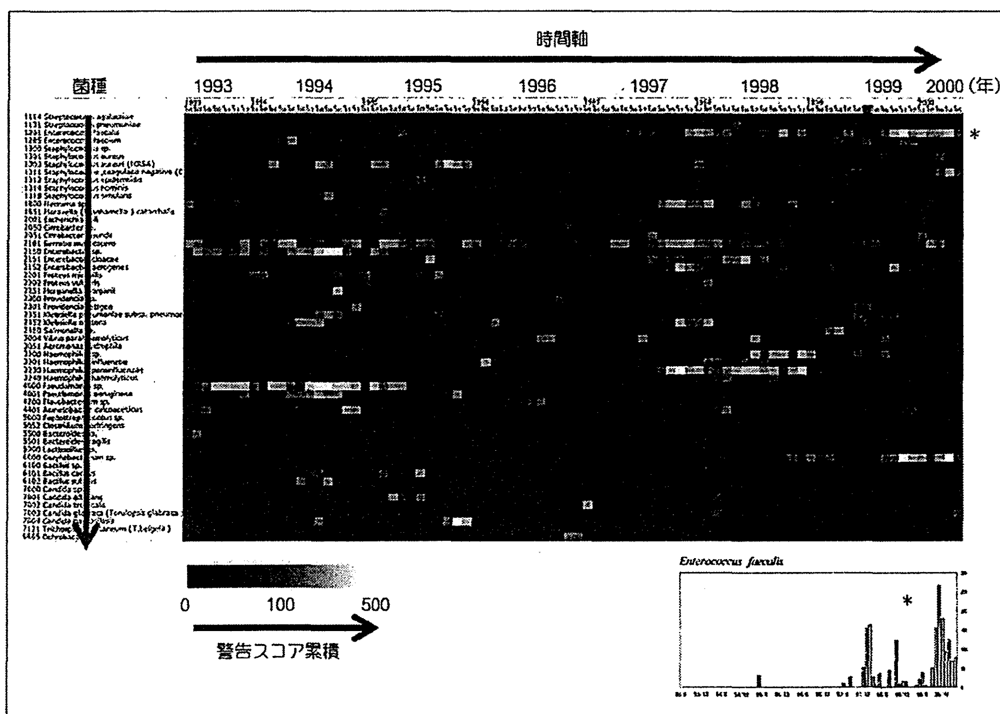

図4 警告スコア累積カラーマトリクス Σ-alert matrix

横軸は時間，縦軸は菌種を示している。四角の色はその菌のその月の PMA の警告スコアの累積に対するカラースケールであり，Σ-alert の棒グラフの高さをカラースケール化したものである。右下の Σ-alert の図に対応する行に＊印を付した。この図ではカラースケールの代わりにグレイスケールを用いている。この例では 1995 年の中頃～ 1997 年前半にかけて一時的に菌の院内拡散が減少している（白い部分が減っている）ことがわかるが，この施設では病棟の増築工事を 1994 年の 12 月～ 1996 年の4月まで行い，一時的に入院患者数を制限していたことがわかっている。それ以外の期間は明るい部分が多く，相当数の院内拡散があることがわかる。

PMA：菌の異常集積の自動検出

（筆者作成）

出に必要な処理のシステム負担が大きいため現実的には難しい。PMA の算出を簡便に行う方法について検討している。

3．2DCM (Two-dimensional color-coded Carrier Mapping system)

PMA, Σ-alert, Σ-alert matrix は菌の院内拡散を検出する方法，あるいは，その結果を用いて施設内における菌の院内拡散の状況を把握するための方法である。これに対して 2DCM は個々の菌が院内で分布している状況を，分離時期，場所（病棟あるいは診療科など），患者，患者の移動，菌の感受性パターンによる分類と結びつけて２次元平面に表示する方法であり，PMA によって見出された異常が本当に施設内での拡散かどうかを予測する方法である[7]。

同菌種の複数の菌が分離された場合，同じ株であるかどうかを判断する gold standard はパルスフィールド電気泳動などの分子疫学的方法である。感受性パターンによる株の判定は分子疫学的方法に較べると分解能は低いが，アウトブレイクなど特定株が広がった場

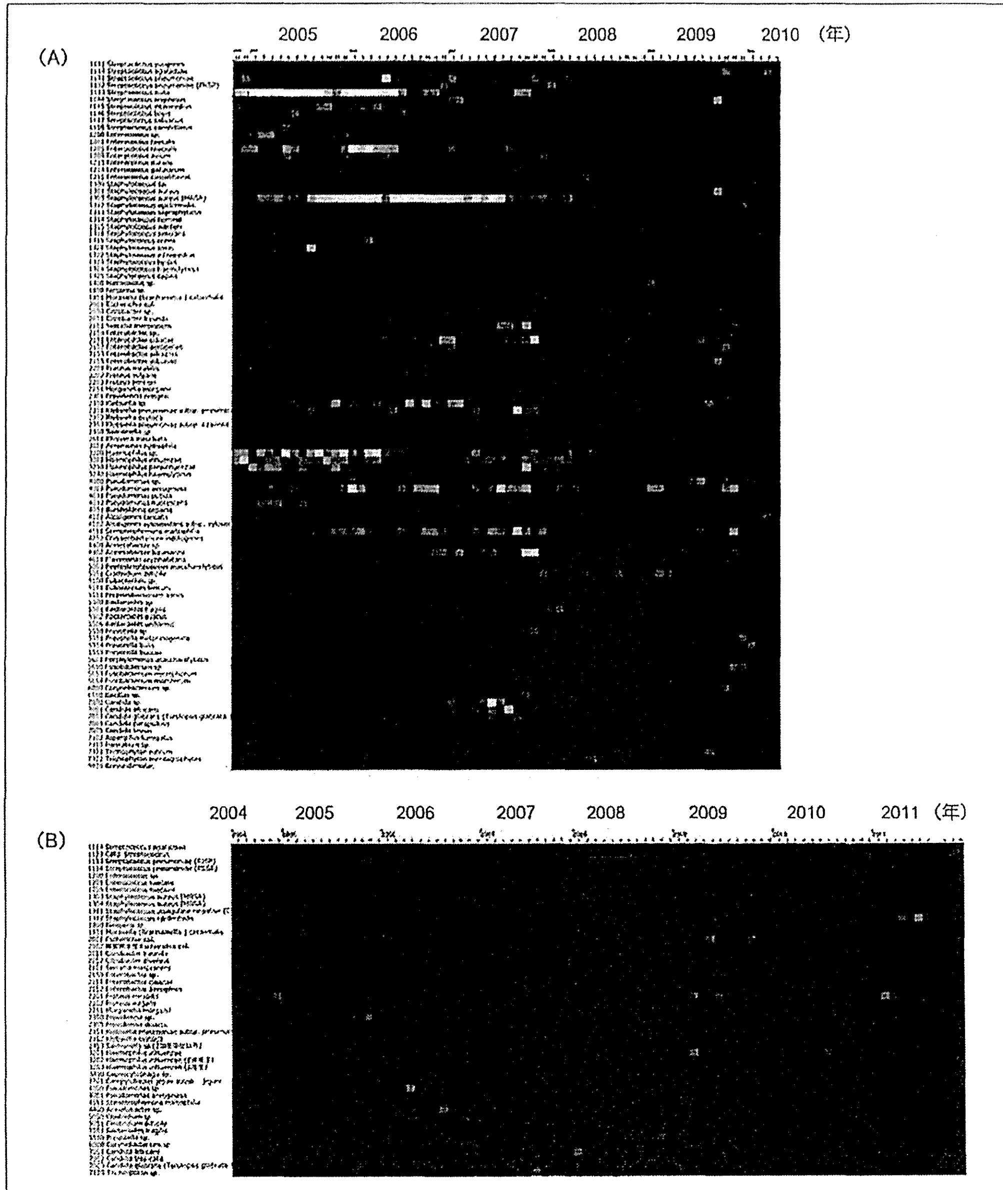

図5　Σ-alert matrix の例

（A）MRSA などの院内感染をくり返していた大学病院。2008 年後半から菌の院内拡散が減少。2008 年頃から院内感染対策の強化が行われていた。2008 年までの左 5/3 程度には明るく見える菌の院内拡散が多く記録されているが，その後の時期では明るい部分が減り，菌の院内拡散が減っていることがわかる。

（B）感染対策の行き届いていた中規模病院。2008 年の後半から菌の院内拡散が増加。同時期に研修医の採用をはじめていた。もともと菌の院内拡散が少なく，暗い図であるが，2008 年の後半からわずかであるが明るい部分が増えて，菌の院内拡散が増えたことがわかる。本図ではカラースケールの代わりにグレイスケールを用いている。

MRSA：メチシリン耐性黄色ブドウ球菌　（筆者作成）