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ORIGINAL ARTICLE

Nosocomial infection caused by vancomycin-susceptible multidrug-resistant *Enterococcus faecalis* over a long period in a university hospital in Japan

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ABSTRACT

Compared with other developed countries, vancomycin-resistant enterococci (VRE) are not widespread in clinical environments in Japan. There have been no VRE outbreaks and only a few VRE strains have sporadically been isolated in our university hospital in Gunma, Japan. To examine the drug susceptibility of *Enterococcus faecalis* and nosocomial infection caused by non-VRE strains, a retrospective surveillance was conducted in our university hospital. Molecular epidemiological analyses were performed on 1711 *E. faecalis* clinical isolates collected in our hospital over a 6-year period [1998–2003]. Of these isolates, 1241 (72.5%) were antibiotic resistant and 881 (51.5%) were resistant to two or more drugs. The incidence of multidrug resistant *E. faecalis* (MDR-Ef) isolates in the intensive care unit increased after enlargement and restructuring of the hospital. The major group of MDR-Ef strains consisted of 209 isolates (12.2%) resistant to the five drug combination tetracycline/erythromycin/kanamycin/streptomycin/gentamicin. Pulsed-field gel electrophoresis analysis of the major MDR-Ef isolates showed that nosocomial infections have been caused by MDR-Ef over a long period (more than 3 years). Multilocus sequence typing showed that these strains were mainly grouped into ST16 (CC58) or ST64 (CC8). Mating experiments suggested that the drug resistances were encoded on two conjugative transposons (integrative conjugative elements), one encoded tetracycline-resistance and the other erythromycin/kanamycin/streptomycin/gentamicin-resistance. To our knowledge, this is the first report of nosocomial infection caused by vancomycin-susceptible MDR-Ef strains over a long period in Japan.

Key words *Enterococcus faecalis*, multidrug resistance, non-vancomycin-resistant enterococci, nosocomial infection.

The incidence of *Enterococcus* infections is increasing and this organism has become a significant cause of nosocomial infections worldwide (1). *Enterococcus faecalis* and *Enterococcus faecium*, commonly isolated from humans, account for 85–95% and 5–10%, respectively, of the enterococcal strains isolated from clinical infections (2). Many clinical enterococcal isolates exhibit multidrug resistance, providing these organisms with a selective advantage in the hospital environment. Outbreaks of nosocomial infections caused by enterococci resistant to various drugs have been reported in Europe and the USA (3, 4). Since the first isolation of VRE in Europe, they have spread and been found more frequently both in environments (e.g. food animals) and

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List of Abbreviations: ABPC, ampicillin; CC, clonal complex; CP, chloramphenicol; Ef, *Enterococcus faecalis*; EM, erythromycin; GM, gentamicin; ICE, integrative conjugative element; ICU, intensive care unit; KM, kanamycin; MDR, multidrug resistance; MIC, minimal inhibitory concentration; MLST, multiple locus sequence typing; PFGE, pulsed field gel electrophoresis; SM, streptomycin; ST, sequence type; TC, tetracycline; VRE, vancomycin resistant enterococci.

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in hospitals throughout the world (5, 6). Outbreaks and nosocomial infection caused by VRE pose a serious clinical problem in many developed countries (1, 6).

In Japan, we have reported nosocomial infections caused by high-level gentamicin-resistant *E. faecalis* (MIC > 500 mg/L) in Gunma University Hospital (7). In 1996, we also reported the first isolation of VRE from a patient in Japan (8). The incidence of isolation of VRE is increasing in Japan; however, according to the Japan Nosocomial Infections Surveillance (9), VRE are not widespread in the hospital environment compared with other developed countries. There are still few reports of VRE or nosocomial infections caused by multidrug resistant enterococcal strains in Japan (10).

In this study, we report a retrospective surveillance of *E. faecalis* infections over 6 years [1998–2003] in Gunma University Hospital, Japan and provide evidence of occurrence over a long period (more than 3 years) of nosocomial infection caused by vancomycin-susceptible, MDR-Ef clones. We also demonstrate the roles of conjugative transposons (ICE) and pheromone-responsive plasmids in the spread of enterococcal drug resistance.

MATERIALS AND METHODS

Bacteria, media and reagents

Clinical isolates ($n = 1711$) of *E. faecalis* were obtained from inpatients at Gunma University Hospital between 1998 and 2003 and kept frozen. API Strep 20 (bioMerieux, Durham, NC, USA) was used for the identification of *E. faecalis*. Media used were Todd–Hewitt broth (Difco Laboratories, Detroit, MI, USA) and Mueller–Hinton broth (Nissui, Tokyo, Japan). The MICs to the antibiotics were determined by an agar dilution method using Mueller–Hinton agar plates. Overnight cultures of the stains were diluted 100fold with fresh broth. One loopful of each dilution (around 10^4 CFU) was plated on agar plates containing drugs. The plates were incubated for 18 hr at 37°C. For quality control, strain *E. faecalis* ATCC 29212 was used in this study, as recommended by the Clinical Laboratory Standard Institute performance standards (M100-S17). Throughout this study, the breakpoints of MICs for "resistance" to antibiotics were defined as follows (mg/L): TC, >12.5; EM, >12.5; KM, >500; SM, >500; GM, >500; CP, >12.5; ABPC, >12.5; and VCM, >3 (Table 1).

Detection of drug resistance genes

The genes *erm*(B), *tet*(M), *lin*(B), *aph(3')-IIIa*, *ant(4")-Ia*, *ant(6')-Ia*, *aac(6')-Ie-aph(2")-Ia*, *aph(2")-Ib*, *aph(2")-Ic*, *aph(2")-Id*, *acc(6')-Ii* and *ant(9)-Ia* were examined by PCR using specific primer sets as described elsewhere (11). The amplified PCR products were confirmed by DNA sequence analysis.

Isolation and manipulation of plasmid DNA

Plasmid DNA was isolated by an alkaline lysis method (12). Plasmid DNA was treated with restriction endonucleases and analyzed by agarose gel electrophoresis (13).

Mating procedures

Broth and filter matings were performed as described previously with a donor/recipient ratio of 1:10 (7). The concentrations of the antibiotic drugs in the selective agar plates were as follows (mg/L): TC, 12.5; EM, 12.5; KM, 500; SM, 500; and GM, 500.

PFGE of chromosomal DNA

Slices of agarose plugs containing chromosomal DNA were placed into 300 μL of reaction buffer with 50 U of *Sma*I (New England BioLabs, Ipswich, MA, USA), and incubated at 25°C overnight. After digestion, the slices were placed in wells of a 1.2% SeaPlaque GTG agarose gel (FMC, Rockland, ME, USA) and electrophoresed within a clamped homogeneous electric field (CHEF-DR II; Bio-Rad Laboratories, Hercules, CA, USA).

MLST

MLST analysis was performed as previously described (14). The housekeeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yqiL* were analyzed using data from the MLST web site (15).

Clumping assay

Detection of mating aggregation (clumping) was performed as described previously (7). The synthetic pheromones (100 ng/mL) cAD1, cPD1, cCF10, cAM373 and cOB1 were used to determine the specificity of pheromone responses (16).

Reorganization of the tertiary university hospital of this study

In January 2002, our hospital was remodelled and new wards constructed. The renovated hospital consists of large clinical care specialty departments, which results in concentration of patients with similar conditions in the same ward. Three traditional internal medicine departments (Internal Medicine I–III), which each had their own ward, were reorganized into seven new wards

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(Endocrine/Diabetes, Gastrointestinal, Hepatic Metabolism, Cardiovascular, Respiratory/Allergy, Renal/Rheumatism and Hematology). Two traditional surgical departments (Surgery I and II), each of which had its own ward, were reorganized into six new wards (Breast/Endocrine Surgery, Gastrointestinal Surgery, Respiratory Surgery, Pediatric Surgery, Cardiovascular Surgery and Transplantation). The old ICU ward, which had eight beds and before the reorganization mainly served as a post-operative recovery room for cardiovascular surgery, was expanded: the modern centralized ICU ward now has 30 beds. The inpatients are moved between the ICU and general wards depending on the severity of their illness and other needs.

Statistical analysis

Data were processed using the SPSS scientific package SPSS 12.0 (SPSS, Chicago, IL, USA). The statistical significance of findings was evaluated by X^2 and Fisher exact tests. Results were considered to be statistically significant at *P* values < 0.05.

RESULTS

Drug resistances of *E. faecalis* clinical isolates

During the 6 year study period [1998–2003], 1711 *E. faecalis* clinical isolates were obtained and examined using eight antibiotics (Table 1). The sources of specimens and numbers of isolates were as follows: urine, 714 (41.7%); sputum, 309 (18.1%); vaginal swab, 166 (9.7%); exudates, 160 (9.4%); pus, 153 (8.9%); decubitus, 75 (4.4%); blood, 51 (3.0%); bile, 37 (2.2%); and others or unknown, 46 (2.7%). Most *E. faecalis* strains were isolated from immunocompromised patients such as those undergoing chemotherapy for malignant tumors, post-operative inpatients, or those with diabetics. In most cases, the *E. faecalis* isolates were considered to have caused infection; however, limited clinical data were available for this retrospective bacteriological study.

Only 470 strains (27.5%) were susceptible to all antimicrobials tested in this study (Table 1). The remaining 1241 isolates (72.5%) were drug resistant, the resistances and numbers of isolates being as follows: TC, 1111 (64.9%); EM, 769 (44.9%); KM, 731 (42.7%); SM, 518 (30.3%); GM, 485 (28.3%); and CP, 256 (15.0%). Neither ampicillin nor vancomycin resistant strains were isolated. The annual incidences of strains resistant to each drug did not change significantly during the 6 year surveillance period.

The drug resistance patterns are listed in Table 2. Multiple resistance (to two or more antibiotics) was shown by 881 strains (51.5%). A five-drug resistance pattern (TC/EM/KM/SM/GM), shown by 209 isolates (12.2%), accounted for the largest category of MDR-Ef isolates.

In January 2002, the hospital underwent reconstruction and reorganization. With an increase in bed numbers in the ICU ward from eight to 30, *E. faecalis* isolation from patients in the ICU increased dramatically from 82 to 210 isolates (Table 3). The incidences of MDR-Ef (resistance to three or more drugs) in the ICU ward were always much higher than in the rest of the hospital ($P < 0.05$). The incidences in the ICU ward increased significantly after the reorganization, as shown in Table 3 ($P < 0.05$).

PFGE analysis of TC/EM/KM/SM/GM-resistant MDR-Ef isolates

We wanted to investigate the spread of MDR-Ef strains in our hospital and nosocomial infections caused by vancomycin-susceptible *E. faecalis* strains. We therefore focused on the major group of MDR-Ef isolates that were

Table 1. Frequencies of isolation of drug resistant *E. faecalis* clinical strains

	Number of isolates (%) in each year						
Drug (MIC, mg/L)	1998	1999	2000	2001	2002	2003	Total
TC (>12.5)	133 (77.3)	184 (70.0)	212 (68.6)	166 (56.3)	237 (59.3)	179 (64.9)	1,111 (64.9)
EM (>12.5)	79 (45.9)	132 (51.0)	146 (47.2)	120 (40.7)	188 (47.0)	104 (37.7)	769 (44.9)
KM (>500)	83 (48.3)	114 (44.0)	128 (41.4)	115 (39.0)	158 (39.5)	133 (48.2)	731 (42.7)
SM (>500)	58 (33.7)	109 (42.1)	92 (29.8)	73 (24.7)	101 (25.3)	85 (30.8)	518 (30.3)
GM (>500)	59 (34.3)	93 (35.9)	94 (30.4)	62 (20.0)	103 (25.8)	74 (26.8)	485 (28.3)
CP (>12.5)	18 (10.5)	55 (21.2)	60 (19.4)	28 (9.5)	53 (13.3)	42 (15.2)	256 (15.0)
ABPC (>12.5)	0	0	0	0	0	0	0
VCM (>3)	0	0	0	0	0	0	0
Susceptible	36 (20.9)	64 (24.7)	70 (22.7)	98 (33.2)	135 (33.8)	67 (24.3)	470 (27.4)
Total	172	259	309	295	400	276	1,711

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Table 2. Drug resistance patterns of *E. faecalis*

Resistance pattern		Number of isolates (%)		
One drug	TC	321	(18.8)	360 (21.1)
	EM	21	(1.2)	
	KM	9	(0.5)	
	SM	5	(0.3)	
	CP	4	(0.2)	
Two drugs	TC/EM	69	(4.0)	138 (8.1)
	KM/GM	23	(1.3)	
	TC/KM	13	(0.8)	
	TC/SM	12	(0.7)	
	TC/CP	11	(0.6)	
	EM/KM	6	(0.4)	
	EM/CP	2	(0.1)	
	EM/SM	1		
	KM/SM	1		
Three drugs	TC/EM/KM	43	(2.5)	163 (9.5)
	TC/EM/CP	30	(1.8)	
	EM/KM/GM	27	(1.6)	
	TC/EM/SM	13	(0.8)	
	EM/KM/SM	13	(0.8)	
	TC/KM/SM	11	(0.6)	
	TC/KMGM	11	(0.6)	
	KM/SM/GM	8	(0.5)	
	TC/SM/CP	4	(0.2)	
	EM/SM/GM	2	(0.1)	
	EM/KM/CP	1		
Four drugs	TC/EM/KM/GM	66	(3.9)	220 (12.9)
	TC/EM/KM/SM	65	(3.8)	
	TC/EM/KM/CP	32	(1.9)	
	TC/KM/SM/GM	25	(1.5)	
	TC/EM/SM/CP	15	(0.9)	
	TC/EM/SM/GM	11	(0.6)	
	EM/KM/SM/CP	4	(0.2)	
	TC/KM/SM/CP	1		
	EM/KM/GM/CP	1		
Five drugs	TC/EM/KM/SM/GM	209	(12.2)	295 (17.3)
	TC/EM/KM/SM/CP	49	(2.9)	
	TC/EM/KM/GM/CP	33	(1.9)	
	TC/KM/SM/GM/CP	2	(0.1)	
	EM/KM/SM/GM/CP	2	(0.1)	
Six drugs	TC/EM/KM/SM/GM/CP			65 (3.8)
Drug susceptible				470 (27.5)
Total		1711 (100%)		

resistant to five drugs (TC/EM/KM/SM/GM). Of the 209 TC/EM/KM/SM/GM resistant MDR-Ef isolates, 105 strains isolated during certain periods representing both before and after hospital restructuring were chosen and further examined at the molecular level (Fig. 1). Of the 105 strains examined, 51 isolates were obtained during the 11 months between October 1999 and August 2000 (before the restructure) and 54 during the 20 months between August 2001 and May 2003 (after the restructure). Of the 105 isolates, 100 strains were isolated from inpatients more than 3 days after their admission (mostly more than 1 month after admission). The remaining five strains (strain numbers 9, 13, 20, 49, and 52, shown in Fig. 1) were isolated from outpatients who had been inpatients of our university hospital. Chromosomal DNA was examined by PFGE (Fig. 1) and plasmid DNA was also examined (data not shown). The PFGE profiles were classified into several groups, using combinations of letters and numbers. The same letter indicates a similar pattern, suggesting an identical origin or closely related strains. The same letter with a different number shows a small change (one to three bands shift), suggesting that the strains are genetically related. Capital letters indicate multiple isolations from different inpatients, and lower-case letters indicate a single isolation. Isolates showing unique profiles were not grouped and are non-typed (blank), suggesting they are unrelated to the others. Based on PFGE profiles, five groups were identified and designated with letters (A through E) followed by numbers (1 through 9) (Fig. 1). Identical or very similar MDR-Ef strains were isolated from different inpatients and from a variety of wards during this period covered by the study. For example, 28 "A1-type" strains were isolated from 16 patients in 11 different wards (ICU, neonatal intensive care unit, first internal medicine ward, south floors 4, 6 and 8, east floor 4, west floors 4–7) from October 1999 to February 2003. Other types were also multiply isolated from different inpatients (Fig. 1). These findings suggest that several MDR-Ef clones have repeatedly caused nosocomial infections in the hospital over a long period.

MLST analysis of the MDR-Ef isolates

The isolates persistently causing nosocomial infections were further analyzed by MLST (Table 4). The "A-types" (A1, A2) were categorized as ST16 (CC58). The "B-types" (B1–B3), "C-types" (C1–C4) and "D-type" were all categorized as ST64 (CC8). The remaining "E-type" was categorized as ST30. The MLST data also confirmed that nosocomial infections persistently isolated over the long-term were caused by two major MDR-Ef clones, ST16 (CC58) and ST64 (CC8). ST16 strains, including two main types (A1, A2) and eight subtypes (a1–a8), were isolated from 26 patients in 17 different wards, whereas ST64 strains, including eight main types (B1–3, C1–4 and D) and 17 subtypes (b1–b8, c1–c9) were isolated from 39 patients in 14 different wards. These results suggest that both clones have become established in the hospital environment and have repeatedly caused nosocomial infections during the period analyzed.

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Table 3. Incidence of MDR-Ef in the ICU

MDR-Ef	1998–2001 (before reconstruction)				2002–2003 (after reconstruction)			
	Entire hospital (600 beds)	Incidence (/bed × year)	Old ICU (eight beds)	Incidence (/bed × year)	Entire hospital (650 beds)	Incidence (/bed × year)	New ICU (30 beds)	Incidence (/bed × year)
three drugs≦	453 (43.8%)	0.189	33 (40.2%)	1.03†	290 (42.9%)	0.223	94 (44.8%)	1.57†,‡
four drugs≦	368 (35.6%)	0.153	27 (32.9%)	0.84†	212 (31.4%)	0.163	75 (35.7%)	1.25†,‡
five drugs≦	224 (21.6%)	0.102	15 (18.3%)	0.46†	136 (20.1%)	0.105	45 (21.4%)	0.75†,‡
Total isolates	1035 (100%)	0.431	82 (100.0%)	2.56†	676 (100%)	0.52	210 (100%)	3.5†,‡

†, the incidences in ICU were higher than those in the rest of the hospital ($P < 0.05$); ‡, the incidences in the new ICU were significantly higher than those in the old ICU ($P < 0.05$).

Experimental conjugation study of MDR-Ef clones

To examine the localization of resistance genes and investigate the roles of plasmids, experimental *in vitro* conjugation studies were performed using five representative MDR-Ef clones (strains No. 2, 8, 84, 85 and 98 [grouped as ST16 and A-types in this study]) (17). All these strains displayed induced mating aggregation in response to synthetic peptide cCF10, indicating that they carried pCF10-type pheromone-responsive plasmids (7, 13). No drug-resistant transconjugants were obtained by broth mating, suggesting either that the five resistance genes are not linked to (encoded on) the pheromone-responsive plasmid, or that the plasmid has lost the ability to transfer. Filter mating experiments resulted in transconjugants on all of the selective plates: representative data for two strains (strains No. 82 and 98) are shown in Table 5. The data from the mating experiments and plasmid profiles indicated that four resistances (EM/KM/SM/GM) were transferred together and that TC-resistance transferred independently. These findings suggest that the MDR-Ef strains carry two conjugative transposons (ICEs, one conferring EM/KM/SM/GM-resistance and the other TC-resistance (18, 19).

Detection of drug resistance genes in a MDR-Ef clone

One of the clonal MDR-Ef strains (strain No. 98) was examined for drug resistance genes by PCR. The drug resistance genes *tet*(M), *erm*(B), *lin*(B), *aac(6')-Ie-aph-(2")-Ia*, *ant(6')-Ia*, *sat(4)* and *aph(3')-IIIa* were detected (data not shown). Combining these findings with the conjugation data, four aminoglycoside resistance genes and an erythromycin resistance gene could be encoded on an ICE, and a tetracycline resistance gene could be encoded on another ICE. The presence of resistance genes on the chromosome was confirmed by Southern hybridization analysis using specific probes (data not shown).

DISCUSSION

In this survey, VRE were not isolated in our hospital between 1998 and 2003; however, most of the isolates were MDR-Ef strains. Although the first VRE were isolated in Japan in 1998, isolation of VRE from clinical sources still rarely occurs compared with other countries (8, 10). Since November 1991, vancomycin has mostly been used to treat methicillin-resistant *Staphylococcus aureus* infections in Japan. The amount of vancomycin used in Japan remains fairly low compared with Europe and the USA (10), which may be one reason for the infrequent isolation of VRE in Japan. However, there have been reports of VRE isolation from imported chicken meat samples in Japan (20). In our nationwide surveillance, VRE strains were isolated from healthy people, suggesting that such strains may have already spread throughout the general community in Japan (20, unpublished data). If this is the case, more frequent VRE clinical isolates may be inevitable in the near future. Glycopeptide agents, including vancomycin and teicoplanin, must be used judiciously, especially when treating patients with a risk of VRE colonization. Once a patient colonized with VRE is admitted to a hospital and handled improperly, nosocomial VRE infections could occur, causing a serious problem in that hospital.

Our group has reported nosocomial infections caused by high-level gentamicin-resistant *E. faecalis* strains in our hospital in 1998 (7). We described inter-ward transmission of enterococcal strains and found that pheromone-responsive plasmids played a role in dissemination (13). After notification of the risk of nosocomial infection caused by non-VRE strains, standard precautions must be followed more strictly; the staff in our university hospital have therefore been thoroughly educated concerning infection control. However, because most people pay little attention to non-VRE isolates, strict contact precautions against enterococcal infection caused by MDR-Ef strains are rarely practiced in hospitals in Japan.

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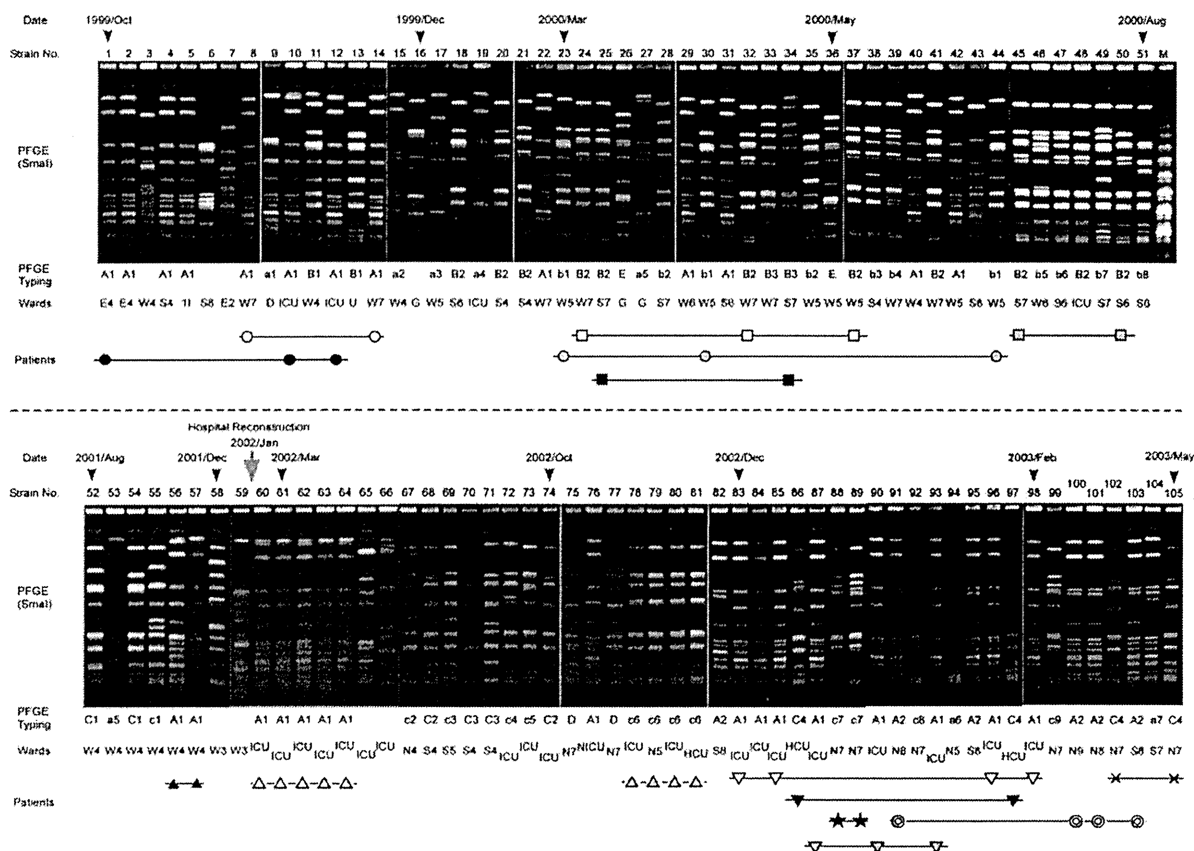

Fig. 1. PFGE analysis (*SmaI*) **of the major multidrug (TC/EM/SM/KM/GM) resistance in *E. faecalis* isolates.** The upper column shows the 51 isolates obtained during the 11 months between October 1999 and August 2000. The lower column shows the 54 isolates obtained during the 20 months between August 2001 and May 2003. Dates on arrowheads are representative isolation dates, shown as a time series. A gray arrow shows the time of hospital reconstruction in January 2002. M indicates a lambda DNA molecular size marker. PFGE profiles are classified into several groups using combinations of letters and numbers, the significance of which is described in the text. Wards/patients indicate the inpatient wards. The abbreviations are as follows: Each horizontal line with symbols indicates repeated isolations from the same patient during hospitalization. Some inpatients changed wards. 1I, first internal medicine ward; D, dermatology ward; E2, east 2nd floor; E4, east 4th floor; G, gynecology ward; HCU, high care unit; ICU, intensive care unit; N4, north 4th floor; N5, north 5th floor; N7, north 7th floor; NICU, neonatal intensive care unit; S4, south 4th floor; S5, south 5th floor; S6, south 6th floor; S7, south 7th floor; S8, south 8th floor; U, urology ward; W3, west 3rd floor; W4, west 4th floor; W5, west 5th floor; W6, west 6th floor; W7, west 7th floor.

In the present study, we retrospectively investigated MDR-Ef isolates (non-VRE) and showed that the major category had a five-drug (TC/EM/SM/KM/GM) resistance pattern. The results of PFGE analysis suggest that TC/EM/SM/KM/GM resistant MDR-Ef strains have repeatedly caused nosocomial infections in our hospital over a long period. However, we cannot completely rule out the possibility of carry-in infections caused by similar independent MDR-Ef strains in this retrospective study. The PFGE patterns of *E. faecalis* in the general population are quite heterogeneous, especially regarding drug sensitive strains, and maybe also for highly resistant strains (21, 22), which supports our conclusion that nosocomial infections and/or nosocomial transmissions are caused by the same MDR-Ef strains within our hospital.

Isolates in this majority group were mainly classified as ST16 (CC58) or ST64 (CC8) strains by MLST. VRE isolates grouped as ST16 (CC58) have been reported in some European countries, including Spain, Poland and the Netherlands (14, 23), and VRE isolates grouped as ST64 (CC8) have been detected in the USA (6, 23). Our data suggest that these two *E. faecalis* clones, independent of drug resistances, might be adapted to colonizing the human intestine globally. The human-adapted clones may have acquired drug resistance genes from other organisms. Drug resistances, including vancomycin resistance, could subsequently be acquired through

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Table 4. MLST of the representative multidrug (TC/EM/SM/KM/GM) resistant *E. faecalis* isolates

Strain No.	Strain name	PFGE typing	*gdh*	*gyd*	*pstS*	*gki*	*aroE*	*xpt*	*ygiI*	ST	CC
2	Ef3290	A1	5	1	1	3	7	7	6	16	58
11	Ef3322	B1	10	1	11	6	5	1	4	64	8
18	Ef3388	B2	10	1	11	6	5	1	4	64	8
26	Ef3487	E	7	1	11	1	10	2	1	30	30
33	Ef3540	B3	10	1	11	6	5	1	4	64	8
52	Ef3849	C1	10	1	11	6	5	1	4	64	8
68	Ef4678	C2	10	1	11	6	5	1	4	64	8
70	Ef4702	C3	10	1	11	6	5	1	4	64	8
75	Ef4813	D	10	1	11	6	5	1	4	64	8
82	Ef4905	A2	5	1	1	3	7	7	6	16	58
86	Ef4947	C4	10	1	11	6	5	1	4	64	8
98	Ef5025	A1	5	1	1	3	7	7	6	16	58

The numbers in the gene categories indicate the allele numbers registered on the MLST web site (15).

horizontal gene transfer. Many drug resistances are encoded on transposons and frequently inserted into plasmids or conjugative transposons, resulting in large composite conjugative transposons (ICEs) (18, 19). The five drug resistances of the major MDR-Ef strains are likely to be encoded on conjugative transposons. Four of the five resistances, including gentamicin resistance, are linked and encoded on a composite conjugative transposon on the chromosome.

In January 2002, our hospital was remodeled. The ICU was expanded to serve critically ill patients hospital-wide, about 60% of these patients being post-operative, 20% from hospital wards and 20% from the emergency room. In the restructured ICU, doctors, nurses and clinical engineers treat critically ill patients using life support devices such as ventilators, extracorporeal membrane oxygenation, intra-aortic balloon pumping, ventricular assist devices and plasmapheresis. Contrary to expectations, over the 2 years after the clean modern hospital had been established, the incidence of MDR-Ef strains from the ICU increased (Table 3). The frequency of isolation (incidence per bed and year) of MDR-Ef strains resistant to three or more antibiotics, four or more antibiotics, or five or more antibiotics, rose around 1.5-fold (1.57/1.03, 1.25/0.84 and 0.75/0.46, respectively), and these were statistically significant increases ($P < 0.05$). Thus, the ICU was an important area to target with anti-infection measures. The renovated hospital consists of large clinical care specialty departments that concentrate patients with similar conditions in the same ward, attended to by the same staff, and medicated according to similar guidelines, including with antibiotic therapies. This may facilitate the increase, transmission and spread of drug resistant strains. In particular, because it functions as a hub ward in the hospital, the modern centralized ICU could be a high-risk environment for the rapid and extensive spread of nosocomial infections. Strict infection control measures, including

Table 5. Experimental conjugation study by filter mating and transfer frequency of drug resistances

Strain No.	Strain name	PFGE pattern/ MLST typing	Pheromone-responsive plasmid	Selective Drug	Transfer frequency (transconjugant/donor)	Drug resistance patterns of the ten transconjugants examined (number of strains)†
98	Ef5025	A1-type/ST16 (CC8)	pCF10 type (cCF10)	TC	4.0×10^{-8}	TC (10)
				EM	2.2×10^{-7}	EM/KM/SM/GM (10)
				KM	2.6×10^{-6}	EM/KM/SM/GM (9), TC/EM/KM/SM/GM (1)
				SM	4.4×10^{-7}	EM/KM/SM/GM (9), TC/EM/KM/SM/GM (1)
				GM	4.0×10^{-7}	EM/KM/SM/GM (7), TC/EM/KM/SM/GM (3)
82	Ef4905	A2-type/ST16 (CC8)	pCF10 type (cCF10)	TC	2.6×10^{-7}	TC (10)
				EM	2.8×10^{-6}	EM/KM/SM/GM (6), TC/EM/KM/SM/GM (4)
				KM	9.6×10^{-6}	EM/KM/SM/GM (4), TC/EM/KM/SM/GM (6)
				SM	5.4×10^{-6}	EM/KM/SM/GM (6), TC/EM/KM/SM/GM (4)
				GM	1.2×10^{-6}	EM/KM/SM/GM (8), TC/EM/KM/SM/GM (2)

†, each transconjugant was randomly chosen from one selective plate. Concentrations of the selected drugs (mg/L): TC, 12.5; EM, 12.5; KM, 500; SM, 500; GM, 500.

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contact precautions, may be needed to prevent and control nosocomial infections and transmissions caused by the ignored persistent MDR bacteria identified by this study.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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Bacteriocin Protein $BacL_1$ of *Enterococcus faecalis* Targets Cell Division Loci and Specifically Recognizes L-Ala_2-Cross-Bridged Peptidoglycan

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Bacteriocin 41 (Bac41) is produced from clinical isolates of *Enterococcus faecalis* and consists of two extracellular proteins, $BacL_1$ and BacA. We previously reported that $BacL_1$ protein (595 amino acids, 64.5 kDa) is a bacteriolytic peptidoglycan D-isoglutamyl-L-lysine endopeptidase that induces cell lysis of *E. faecalis* when an accessory factor, BacA, is copresent. However, the target of $BacL_1$ remains unknown. In this study, we investigated the targeting specificity of $BacL_1$. Fluorescence microscopy analysis using fluorescent dye-conjugated recombinant protein demonstrated that $BacL_1$ specifically localized at the cell division-associated site, including the equatorial ring, division septum, and nascent cell wall, on the cell surface of target *E. faecalis* cells. This specific targeting was dependent on the triple repeat of the SH3 domain located in the region from amino acid 329 to 590 of $BacL_1$. Repression of cell growth due to the stationary state of the growth phase or to treatment with bacteriostatic antibiotics rescued bacteria from the bacteriolytic activity of $BacL_1$ and BacA. The static growth state also abolished the binding and targeting of $BacL_1$ to the cell division-associated site. Furthermore, the targeting of $BacL_1$ was detectable among Gram-positive bacteria with an L-Ala-L-Ala-cross-bridging peptidoglycan, including *E. faecalis*, *Streptococcus pyogenes*, or *Streptococcus pneumoniae*, but not among bacteria with alternate peptidoglycan structures, such as *Enterococcus faecium*, *Enterococcus hirae*, *Staphylococcus aureus*, or *Listeria monocytogenes*. These data suggest that $BacL_1$ specifically targets the L-Ala-L-Ala-cross-bridged peptidoglycan and potentially lyses the *E. faecalis* cells during cell division.

Enterococcus faecalis is a commensal Gram-positive bacterium in the intestinal tract of healthy humans or animals and is also known to be an opportunistic pathogen causing various infectious diseases, including urinary infectious disease, bacteremia, infective endocarditis, and others (1–3). The infection-derived *E. faecalis* strains often produce various plasmid-encoded bacteriocins (4, 5).

Bacteriocins are bacterial peptides or proteins with antimicrobial activities (6). Heat- and acid-stable bacteriocin peptides produced by Gram-positive bacteria are divided into class I and class II according to posttranslational modifications (7, 8). Class I bacteriocins are lantibiotics that contain nonproteinogenic amino acids generated by posttranslational modification (9). Only two class I bacteriocins have been identified in enterococci: β-hemolysin/bacteriocin (cytolysin) and enterocin W (10–14). In contrast, most enterococcal bacteriocins belong to class II and are nonmodified antimicrobial peptides, such as AS-48, enterocin A, and others (7, 15, 16). We have found the enterococcal class II bacteriocins, including Bac21, Bac31, Bac32, Bac43, and Bac51, in clinical strains of *E. faecalis* or *Enterococcus faecium* (17–21). Unlike the low-molecular-weight peptide-type class I and II bacteriocins, heat-labile antimicrobial proteins are referred to as bacteriolysins, previously named class III bacteriocins, and show enzymatic bactericidal activity (22, 23). In enterococci, the bacteriolysins enterolysin A and bacteriocin 41 (Bac41) have been identified (24–26).

Bac41 was originally found expressed from the pheromone-responsive plasmid pYI14 carried by the clinical strain *E. faecalis* YI14 (26, 27). The Bac41-type bacteriocins were also found in the *E. faecalis* VanB-type vancomycin-resistant *E. faecalis* (VRE) outbreak strains (27). Bac41 is specifically active only against *E. faecalis* (26, 28). The determinant region of Bac41 contains six open reading frames (ORFs), including *bacL₁*, *bacL₂*, *bacA*, and *bacI* (Fig. 1A). The bactericidal activity of Bac41 is actually expressed by the two extracellular components, the *bacL₁*- and *bacA*-encoded proteins $BacL_1$ and BacA (26). $BacL_1$ and BacA are secreted proteins that coordinately exert bactericidal activity against *E. faecalis* (26, 28). $BacL_2$ positively regulates the transcripts of *bacL₁* and *bacL₂* itself (unpublished data). BacI is the specific immunity factor protecting a Bac41 producer from Bac41 activity (26).

We previously demonstrated that $BacL_1$ is a peptidoglycan D-isoglutamyl-L-lysine endopeptidase (28). $BacL_1$ has 595 amino acids with a molecular mass of 64.5 kDa and consists of two distinct peptidoglycan hydrolase homology domains and three repeats of the SH3 domain (Fig. 1B). The two peptidoglycan hydrolase domains located in the regions from amino acid 3 to 140 and amino acid 163 to 315 show homology to the bacteriophage-type pepti-

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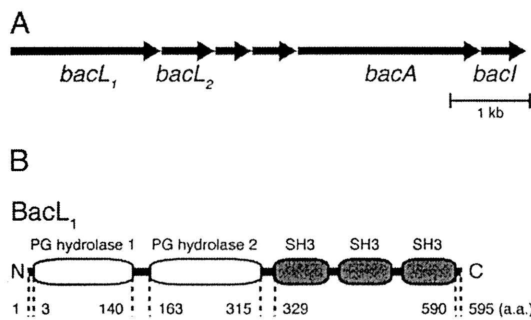

FIG 1 Schematics of Bac41 gene organization and $BacL_1$ structure. (A) Organization of Bac41-related genes (GI 169635857). (B) Molecular structure of $BacL_1$ (GI 169635864). Two domains with homology to distinct peptidoglycan hydrolases, bacteriophage-type hydrolase and NlpC/P60 family hydrolase, are present in the regions from amino acid (a.a.) 3 to 140 and amino acid 163 to 315, respectively. Three repeats of the bacterial SH3 domain are present in the region from amino acid 329 to 590.

doglycan hydrolase and the NlpC/P60 family peptidoglycan hydrolase, respectively (26, 29, 30). The second peptidoglycan hydrolase homologue, with similarity to NlpC/P60, has D-isoglutamyl-L-lysine endopeptidase activity against the purified peptidoglycan component from *E. faecalis* (28). On the other hand, the molecular function of the first peptidoglycan hydrolase domain, with similarity to bacteriophage-type peptidoglycan hydrolase, remains to be elucidated but is still required for the bactericidal activity against viable *E. faecalis* cells (28). The SH3 repeat domain is located in the region from amino acid 329 to 590 and functions as the binding domain to the peptidoglycan (28). However, $BacL_1$ is not sufficient for bactericidal activity. BacA appears to be essential for bactericidal activity, together with $BacL_1$, although its function also remains to be determined (26, 28).

On the basis of cell morphology, enterococci are grouped in the ovococci, whose cell shapes are elongated ellipsoids (31–33). In ovococci, the model of the dividing cell wall assembly process is distinct from that of other shaped bacteria, such as spherical cocci. The cell division of ovococci is achieved by two distinct cell wall-synthesizing machineries that manage peripheral and septal cell wall growth. The peripheral cell wall growth is responsible for the longitudinal cell elongation. On the other hand, the septal cell wall growth occurs to allow splitting into separated daughter cells. In this study, by using fluorescent dye-conjugated recombinant proteins, we demonstrated that $BacL_1$ localized to the cell division-related cell surface of target *E. faecalis* cells and that cell division was required for susceptibility to the bactericidal activity expressed by $BacL_1$ and BacA.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, media, and antimicrobial reagents. The bacterial strains and plasmids used in this study are shown in Table 1. A standard plasmid DNA methodology was used (34). Enterococcal strains were routinely grown in Todd-Hewitt broth (THB; Difco, Detroit, MI) at 37°C (35), unless otherwise noted. *Escherichia coli* strains were grown in Luria-Bertani medium (LB; Difco) at 37°C. Gram-positive bacterial species (other than *Enterococcus*) were grown in brain heart infusion (BHI) medium (Difco) at 37°C. The antibiotic concentrations for the selection of *E. coli* were 100 mg liter^{-1} ampicillin and 30 mg liter^{-1} chloramphenicol. The concentration of chloramphenicol for the routine selection of *E. faecalis* carrying plasmid pAM401 or its derivatives was 20 mg liter^{-1}, unless otherwise noted. All antibiotics were obtained from Sigma Co. (St. Louis, MO).

Recombinant proteins and antibodies. The histidine-tagged recombinant proteins of full-length $BacL_1$, its truncated derivatives, and BacA

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. faecalis OG1S	*str*, derivative of OG1	35
E. faecalis OG1X	*str*, protease-negative derivative of OG1	35
E. faecalis OG1RF	*rif fus*, derivative of OG1	35
E. faecalis FA2-2	*rif fus*, derivative of JH2	60
E. faecium BM4105RF	*rif fus*, derivative of BM4105	61
E. hirae 9790	Type strain	ATCC 9790
S. aureus F-182	Clinical isolate, resistant to methicillin and oxacillin	ATCC 43300
S. pyogenes MGAS315	Clinical isolate, serotype M3	ATCC BAA-595
S. pneumoniae 262	Quality control strain, serotype 19F	ATCC 49619
L. monocytogenes EGD	Serovar 1/2a	ATCC BAA-679
E. coli DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*argE-lacZYA*)*U169*, host for DNA cloning	Bethesda Research Laboratories
E. coli BL21	*ompT hsdSB*(r_B^- m_B^-) *gal*(λcI 857 *ind1 Sam7 nin5 lacUV5-T7gene1*) *dcm*(DE3), host for protein expression	Novagen
Plasmids		
pAM401	*E. coli-E. faecalis* shuttle plasmid; *cat tet*	62
pHT1100	pAM401 derivative containing wild-type Bac41 genes	26
pET22b(+)	Expression plasmid for His-tagged protein in *E. coli*	Novagen
pET::*bacL₁*	pET22b(+) derivative expressing $BacL_1$	28
pET::*bacL₁* Δ1	pET22b(+) derivative expressing $BacL_1$Δ1	28
pET::*bacL₁* Δ2	pET22b(+) derivative expressing $BacL_1$Δ2	28
pET::*bacL₁* Δ1Δ2	pET22b(+) derivative expressing $BacL_1$Δ1Δ2	28
pET::*bacL₁* Δ3	pET22b(+) derivative expressing $BacL_1$Δ3	28
pET::*bacA*	pET22b(+) derivative expressing BacA	28

were prepared by the Ni-nitrilotriacetic acid (NTA) system as previously described (28). The green or red fluorescent dye-labeled recombinant proteins were prepared with NH_2-reactive fluorescein or NH_2-reactive HiLyte Fluor 555 (Dojindo, Kumamoto, Japan), respectively. By performing a soft-agar bacteriocin assay, we confirmed that fluorescent dye-conjugated $BacL_1$ remains active (see Fig. S1 in the supplemental material). Anti-$BacL_1$ antibody was prepared by immunization of rabbits with recombinant $BacL_1$-His protein as previously described (Operon Technologies, Alameda, CA) (28).

Fluorescence microscopy. Bacteria diluted with fresh medium were mixed with fluorescent recombinant protein as indicated and incubated at 37°C for 1 h. The bacteria were collected by centrifugation at 5,800 × *g* for 3 min and then fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. The bacteria were rinsed and resuspended with distilled water and mounted with Prolong gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) on a glass slide. The sample was analyzed by fluorescence microscopy (Axiovert 200; Carl Zeiss, Oberkochen, Germany), and images were obtained with a DP71 camera (Olympus, Tokyo, Japan).

Immunogold TEM. Bacteria in early exponential phase were treated with recombinant $BacL_1$ and BacA as indicated and incubated at 37°C for 1 h. The bacteria were fixed with 3% paraformaldehyde–0.1% glutaraldehyde for 10 min at RT and mounted on an electron microscopy (EM) grid. After fixation, the sample grid was treated with 10-fold-diluted anti-$BacL_1$ antibodies in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) at 37°C for 1 h, followed by a wash with PBS. Then, the grid was treated with 10-fold diluted colloidal gold (15 nm)-conjugated anti-rabbit IgG in PBS containing 2% BSA for 30 min at RT, washed with PBS, and then negatively stained with 2% ammonium molybdate for 1 min at RT. The resulting samples were analyzed by transmission electron microscopy (TEM) (JEM-1010; JEOL Ltd., Tokyo, Japan).

Bacteriolytic assay. The soft-agar assay or liquid-phase assay for bacteriocin activity was performed as described previously (36). Briefly, the test bacterial strain or 1 μl of the recombinant protein solution was inoculated onto THB soft agar (0.75%) containing the indicator strain and was then incubated at 37°C for 24 h. The formation of an inhibitory zone was evaluated as susceptibility to the bacteriocin. For the agar-based bacteriolytic assays using *Streptococcus pyogenes* and *Streptococcus pneumoniae*, the indicator bacteria were spread on agar plates by swab instead of using the soft agar. In this swab method, *E. faecalis* OG1S and *E. faecium* BM4105 RF were used for the positive control and the negative control, respectively. For the liquid-phase bactericidal assay, an overnight culture of the indicator strain was diluted with fresh medium, and then the recombinant proteins were added and the sample was incubated at 37°C. Changes in turbidity were monitored by using a spectrometer (DU730; Beckman Coulter, Fullerton, CA) or microplate reader (Thermo Scientific, Waltham, MA).

Cell wall degradation assay. For the cell wall degradation assay, a cell wall fraction was prepared as described previously, with slight modifications (28, 37). The bacterial culture was collected by centrifugation and rinsed with 1 M NaCl. The bacterial pellet was suspended in 4% SDS and heated at 95°C for 30 min. After rinsing with distilled water four times, the bacterial pellet was resuspended with distilled water and mechanically disrupted with 0.1-mm glass beads (As One, Osaka, Japan) using a FastPrep FP100A (Thermo Scientific, Waltham, MA). After unbroken cells were removed by centrifugation at 1,000 rpm for 1 min, the cell wall fraction in the supernatant was collected by centrifugation at 15,000 rpm for 10 min and was then treated with 0.5 mg ml^{-1} trypsin (0.1 M Tris-HCl [pH 6.8], 20 mM $CaCl_2$) at 37°C for 16 h. The sample was further washed with distilled water four times and was resuspended in 10% trichloroacetic acid (TCA), followed by incubation at 4°C for 5 h, and then given additional washes with distilled water four times (38). Finally, the cell wall fraction was resuspended in PBS and quantified by measuring the turbidity for the cell wall degradation assay. Mutanolysin (Sigma) was used as a positive control for the cell wall degradation enzyme.

RESULTS

$BacL_1$ targets the cell division-associated region on the *E. faecalis* surface via its cell wall binding domain. To investigate the localization of $BacL_1$ on target *E. faecalis* cells, we coincubated *E. faecalis* cells and the recombinant $BacL_1$ labeled with red fluorescent dye in the absence or presence of BacA, followed by analysis using fluorescence microscopy (Fig. 2A and B). A specific localization signal of $BacL_1$ in the midcell was observed independently of BacA (Fig. 2A). Furthermore, the four characteristic localization patterns closely correlated with cell growth division were detected (31, 33, 39). First, the most typical localization signal of $BacL_1$ was detected in the midcell, which corresponds to the equatorial ring (Fig. 2B). The equatorial ring structure of the $BacL_1$ localization in the midcell was clearly recognized by the reconstructed image of fluorescence microscopy analysis (see Movie S1 in the supplemental material). Second, the duplicated equatorial ring structure was detected as the source of the localization signal of $BacL_1$ in the cells initiating elongation prior to cell division. Third, in the cells where the cell division process had progressed further, to formation of the division septum, the localization signal of $BacL_1$ was distributed in the area from the equatorial ring to the division septum, where the cell wall is newly synthesized (nascent cell wall) (32). Furthermore, when cell division was completed, localization at the division septum between separated daughter cells, as well as at the equatorial ring, was detected. In addition, immunogold TEM analysis using anti-$BacL_1$ antibodies in *E. faecalis* cultures treated with $BacL_1$ and BacA also showed the equatorial ring localization of $BacL_1$ (Fig. 2C).

We previously reported that $BacL_1$ binds to peptidoglycan of *E. faecalis* via a C-terminal SH3 triple repeat domain localized in the region from amino acid 329 to 590 (28). To investigate the domain required for the specific targeting, domain deletion derivatives of $BacL_1$ were labeled with green fluorescent dye (Fig. 3A) and mixed with *E. faecalis* cells, followed by analysis of their location signal by fluorescence microscopy (Fig. 3B). $BacL_1\Delta3$, the derivative with deletion of the C-terminal SH3 repeat, failed to localize to the equatorial ring and did not show any detectable signal. In contrast, $BacL_1\Delta1$, $BacL_1\Delta2$, and $BacL_1\Delta1\Delta2$, derivatives with deletion of the phage-type peptidoglycan hydrolase homology domain, NlpC/P60 family peptidoglycan hydrolase homology domain, or both domains, respectively, were targeted to the equatorial ring similarly to wild-type $BacL_1$. These results indicate that the SH3 repeat was sufficient for the targeting to the equatorial ring on the cell surface of *E. faecalis.* Collectively, $BacL_1$ appeared to target the cell division-related cell surface, including the equatorial ring, the division septum, and the nascent cell wall, via its C-terminal SH3 repeat domains.

Cell division is required for the septum targeting of $BacL_1$ and for the cell lysis triggered by $BacL_1$ and BacA. To analyze the involvement of cell division in Bac41 activity, we investigated the relationship of growth phase and susceptibility to Bac41-induced lysis. *E. faecalis* was grown in fresh THB broth, and a mixture of recombinant $BacL_1$ and BacA was added at different points in the growth phases (Fig. 4A). Adding $BacL_1$ and BacA at the start of incubation (time zero) completely inhibited the increase of the bacterial suspension's turbidity (cell growth). When $BacL_1$ and BacA were added at early or mid-exponential phase, bacterial turbidity was also dramatically decreased, indicating that cells were lysed. In contrast, treatment with $BacL_1$ and BacA at the stationary

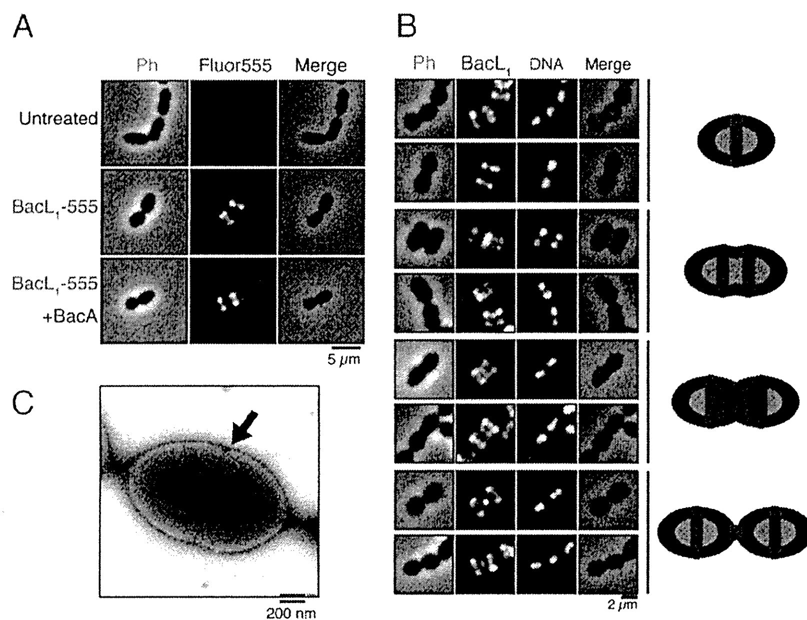

FIG 2 Localization of BacL$_1$ on the *E. faecalis* cell surface. (A) An overnight culture of *E. faecalis* OG1S diluted 5-fold with fresh THB broth was incubated with HiLyte Fluor 555 fluorescent dye-labeled (red) BacL$_1$ (5 μg/ml) in the presence (bottom) or absence (middle) of BacA, followed by analysis using fluorescence microscopy. Bacteria grown without red fluorescent conjugate are also shown as a negative control (top). Phase contrast (Ph) is pseudocolored (green) in the merged image. (B) Extensive representation of the localization pattern of red fluorescent dye-labeled BacL$_1$. The sample preparation was performed exactly as described for panel A. DNA was visualized with DAPI (blue). The schematic on the right illustrates the four characteristic patterns of BacL$_1$ localization and cell division states. (C) *E. faecalis* treated with BacL$_1$ and BacA (5 μg/ml each) was subjected to immunogold transmission electron microscopy using anti-BacL$_1$ antibodies. The arrow indicates the septum localization of gold particles.

phase did not affect the bacterial turbidity, similar to the results for the untreated culture. The bacterial viability test by colony formation assay also indicated that the bactericidal activity of BacL$_1$ and BacA was effective only in early or exponential phase but not stationary phase (Fig. 4B). In contrast, egg white lysozyme was able to decrease the viability of bacteria even in stationary phase (Fig. 4B). These observations indicated that *E. faecalis* in stationary phase was not susceptible to the cell lysis induced by BacL$_1$ and BacA. Then, to test the growth phase dependence of the septum targeting of BacL$_1$, the red fluorescence-labeled BacL$_1$ was incubated with *E. faecalis* cells in early exponential or stationary phase, and the BacL$_1$ localization was analyzed by fluorescence microscopy (Fig. 4C). In the case of the bacteria in early exponential phase, BacL$_1$ localized at the division septum. In contrast, the septum localization of BacL$_1$ was not observed in bacteria in stationary phase. BacL$_1$ also failed to even bind to the cell surface in stationary-phase bacteria (Fig. 4C). These results suggested that BacL$_1$ recognized the dividing cell surface. Furthermore, we investigated the susceptibility to bactericidal activity of BacL$_1$ and BacA when bacterial cell growth was artificially restricted with various antibiotic reagents (Fig. 5). Treatment with bacteriostatic antibiotics, such as chloramphenicol and tetracycline, almost completely rescued the cells from the bacteriolytic activity of BacL$_1$ and BacA (Fig. 5A and B). The localization of BacL$_1$ to the equatorial ring was also abolished in the chloramphenicol- or tetracycline-treated bacteria (Fig. 5C). Treatment with vancomycin, a bactericidal drug blocking cell wall synthesis, resulted in relief of the sensitivity of *E. faecalis* to lysis by BacL$_1$ and BacA and abolished BacL$_1$ targeting to the cell surface (Fig. 5A, B, and C). Interestingly, the bacteria treated with ampicillin, which has an elongating effect on bacterial cells by inhibiting the penicillin binding protein (PBP) functions, appeared to be more susceptible to the bactericidal activity of BacL$_1$ and BacA (Fig. 5A and B) and to the septum targeting of BacL$_1$ (Fig. 5C).

Specific recognition by BacL$_1$ of L-Ala$_2$-type peptidoglycan cross-bridging structure. The composition and length of the cross-bridge peptide-linking stem peptides bound to *N*-acetylmuramic acid are diverse among bacterial species (Fig. 6A) (40, 41). Lu et al. reported that the SH3 domain of ALE-1, a bacteriolytic peptidoglycan hydrolase of *Staphylococcus aureus*, specifically recognizes the pentaglycine cross bridge, which is a specific structure in the peptidoglycan of *S. aureus* (42). As shown by the results in Fig. 3, the SH3 domains appeared to be necessary for targeting the cell division-related region. To investigate whether the SH3 domain of BacL$_1$ also specifically recognizes the cross-bridging structure in the peptidoglycan of *E. faecalis*, we analyzed the cell division-associated targeting of BacL$_1$ in various Gram-positive bacterial species, including *E. faecalis* OG1S, *E. faecalis* OG1X, *E. faecalis* OG1RF, *E. faecalis* FA2-2, *E. faecium* BM4105RF, *Enterococcus hirae* 9790, *S. pyogenes* MGAS315, *S. pneumoniae* 262, *S. aureus* F-182, and *Listeria monocytogenes* EGD (Fig. 6B and Table 2). In bacteria with L-Ala-L-Ala-cross-bridging peptidoglycans, including *E. faecalis* strains OG1S, OG1X, OG1RF, and FA2-2 and *S. pyogenes*, BacL$_1$ clearly localized in the equatorial ring (38, 43–45). In contrast, the BacL$_1$ signal was not detected on *E. faecium*, *E. hirae*, or *S. aureus*, which have L-Asp-, D-Asn-, or penta-Gly-cross-bridging peptidoglycan, respectively (37, 46, 47). *L. monocytogenes*, which has direct bridging between stem peptides, was also not bound with BacL$_1$ (48, 49). In the case of *S. pneumoniae*, which has a hetero-cross-bridging structure consisting of L-Ala-L-Ala

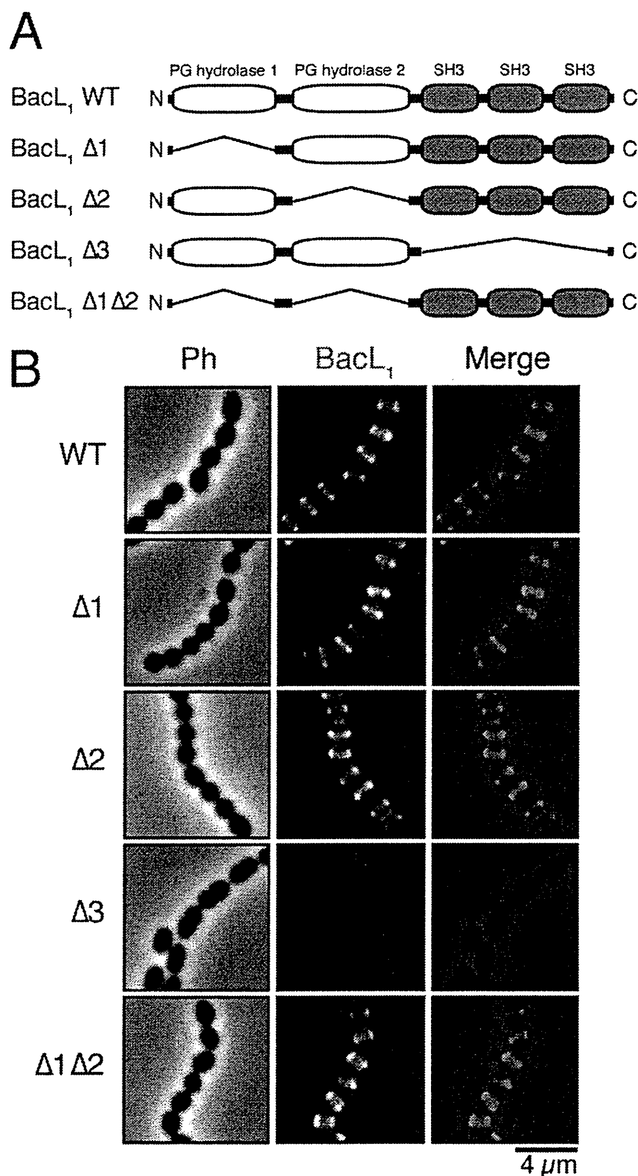

FIG 3 Domain of $BacL_1$ that is responsible for septum targeting. (A) Schematics of truncated $BacL_1$ constructs. (B) Overnight culture of *E. faecalis* OG1S diluted 5-fold with fresh THB broth was incubated with the fluorescein dye-labeled (green) truncated $BacL_1$ proteins (5 μg/ml) depicted in panel A, followed by analysis using fluorescence microscopy. Phase contrast (Ph) is pseudocolored (red) in merged images.

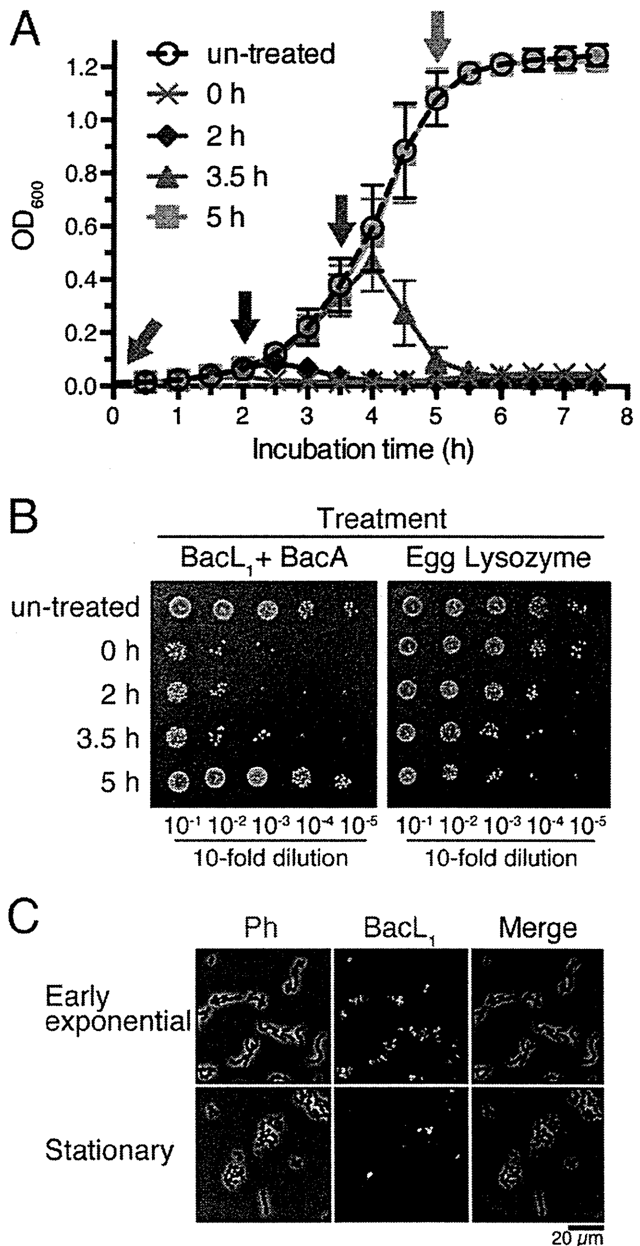

FIG 4 Growth phase dependence of the susceptibility to Bac41. (A) An overnight culture of *E. faecalis* OG1S diluted 100-fold with fresh THB broth was incubated at 37°C. A mixture of recombinant $BacL_1$ and BacA (5 μg/ml each) was added at different growth phases corresponding to 0 h, 2 h, 3.5 h, or 5 h, as indicated with arrows. An untreated culture served as the negative control. The turbidity (optical density at 600 nm [OD_{600}]) was monitored in each culture. The data are presented as the mean results ± standard deviations (SD) of three independent experiments. (B) *E. faecalis* was treated with $BacL_1$ and BacA at different growth phases as described for panel A. After further incubation for 1 h from each time point of addition, the bacterial suspensions were serially diluted 10-fold with fresh THB broth and then spotted onto a THB agar plate, followed by incubation overnight. Colony formation was evaluated as a measure of bacterial viability. Lysozyme was used as a control. (C) *E. faecalis* was treated with HiLyte Fluor 555-labeled (red) $BacL_1$ (5 μg/ml) in the early-exponential (2 h) or stationary (5 h) phase of growth. After further incubation for 1 h from each time point of addition, the cells were fixed and analyzed by fluorescence microscopy. Phase contrast (Ph) is pseudocolored (green) in the merged images.

and L-Ala-L-Ser, the equatorial localization was not observed; however, localization in the division septum was detected (50). Collectively, these observations suggest that $BacL_1$ specifically binds to the L-Ala-L-Ala-cross-bridged peptidoglycan. On the other hand, the bactericidal phenotype of $BacL_1$ and BacA was observed only in *E. faecalis* strains and not in the other bacterial species in soft-agar bacteriocin assays (Table 2). It is notable that *S. pyogenes* and *S. pneumoniae* were not susceptible to $BacL_1$ and BacA despite the targeting of $BacL_1$ to their cell surface (Table 2).

Immunity factor does not alter the $BacL_1$ equatorial targeting. The $BacL_1$- and BacA-producing *E. faecalis* has a self-resis-

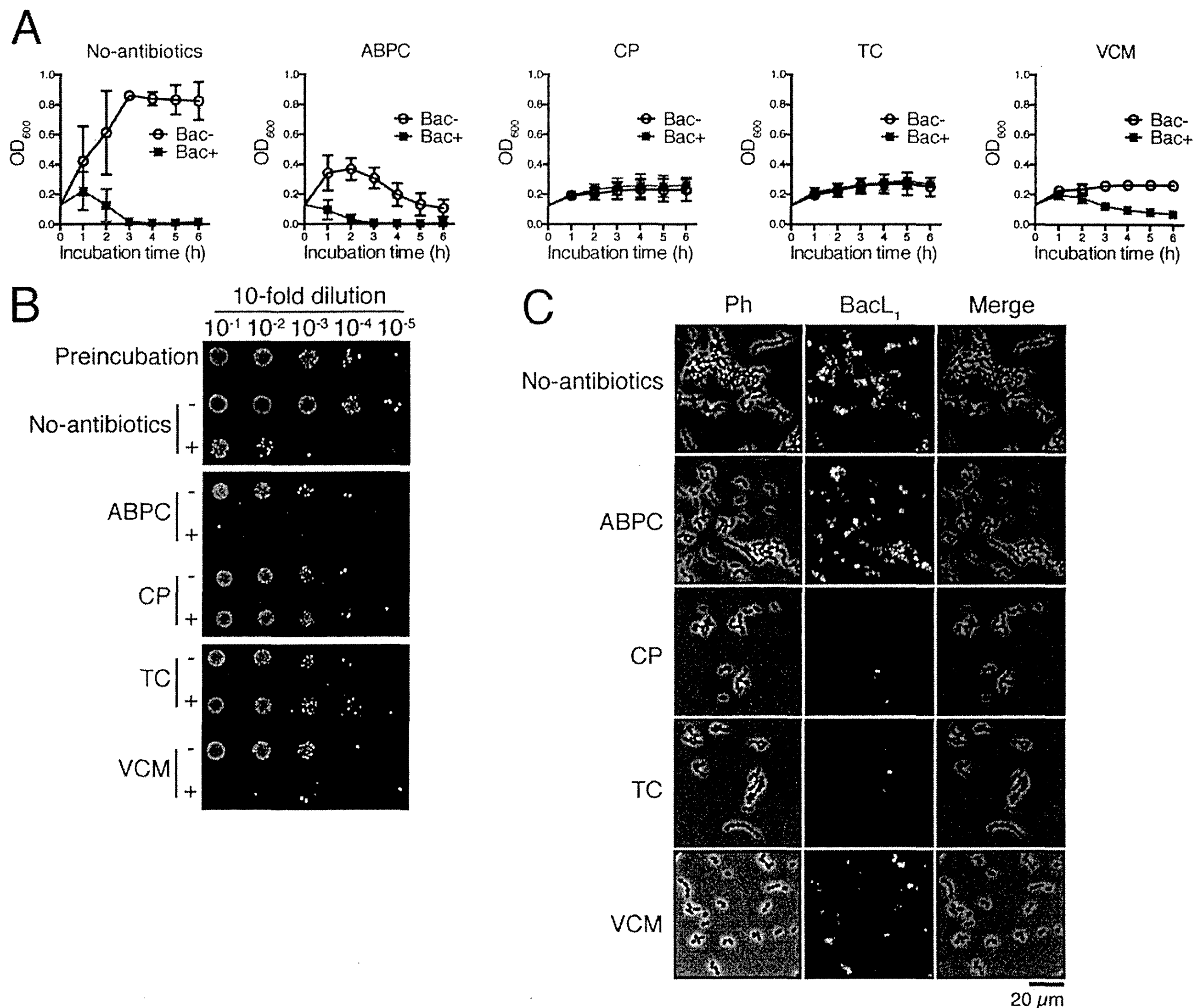

FIG 5 Effects of antibiotics on the susceptibility to Bac41. (A) An overnight culture of *E. faecalis* OG1S diluted 5-fold with fresh THB broth was incubated with (Bac+) or without (Bac−) a mixture of recombinant BacL$_1$ and BacA (5 μg/ml each) in the presence or absence of ampicillin (ABPC; 20 μg/ml), chloramphenicol (CP; 100 μg/ml), tetracycline (TC; 12.5 μg/ml), or vancomycin (VCM; 10 μg/ml). The turbidity at 600 nm was measured with a microplate reader during the incubation period. The data are presented as the mean results ± SD of three independent experiments. (B) *E. faecalis* was treated with (+) or without (−) a mixture of BacL$_1$ and BacA in the presence of antibiotics as described for panel A. After incubation for 6 h, the bacterial suspensions were serially diluted 10-fold with fresh THB broth and then spotted onto a THB agar plate, followed by incubation overnight. Colony formation was evaluated as a measure of bacterial viability. (C) An overnight culture of *E. faecalis* diluted 5-fold with fresh THB broth was treated with HiLyte Fluor 555-labeled (red) BacL$_1$ (5 μg/ml) in the presence of antibiotics as shown. After incubation for 1 h, the cells were fixed and analyzed by fluorescence microscopy. Phase contrast (Ph) is pseudocolored (green) in merged images.

tance factor, BacI, encoded in the vicinity of the *bacA* gene (Fig. 1A) (26). *E. faecalis* carrying the *bacI* gene is completely resistant to the bacteriolytic effect of BacL$_1$ and BacA (26, 28). Therefore, by fluorescence microscopy, we investigated whether the immunity factor *bacI* affects the BacL$_1$ targeting. The equatorial localization of BacL$_1$ was observed in *E. faecalis* carrying pHT1100 (a plasmid containing all Bac41 genes, including immunity factor *bacI*), as well as in *E. faecalis* carrying pAM401 (a vector control without the *bacI* gene) (Fig. 7A). Furthermore, the peptidoglycan purified from *E. faecalis* carrying pHT1100 was still degraded by BacL$_1$ (Fig. 7B). These results suggest that the specific immunity factor, BacI, has no effect on the BacL$_1$ activities of binding, targeting, and degrading peptidoglycan.

DISCUSSION

In this study, we report that BacL$_1$ targets the cell division-associated site, including the equatorial ring, division septum, and nascent synthesized cell wall (Fig. 2), to exert potential bactericidal activity against *E. faecalis* cells in the dividing state (Fig. 4 and 5). We also demonstrate that BacL$_1$ specifically recognizes peptidoglycan structures cross-linked by L-Ala-L-Ala but not by other peptide linkers (Fig. 6). Although the entire cell wall in *E. faecalis* is

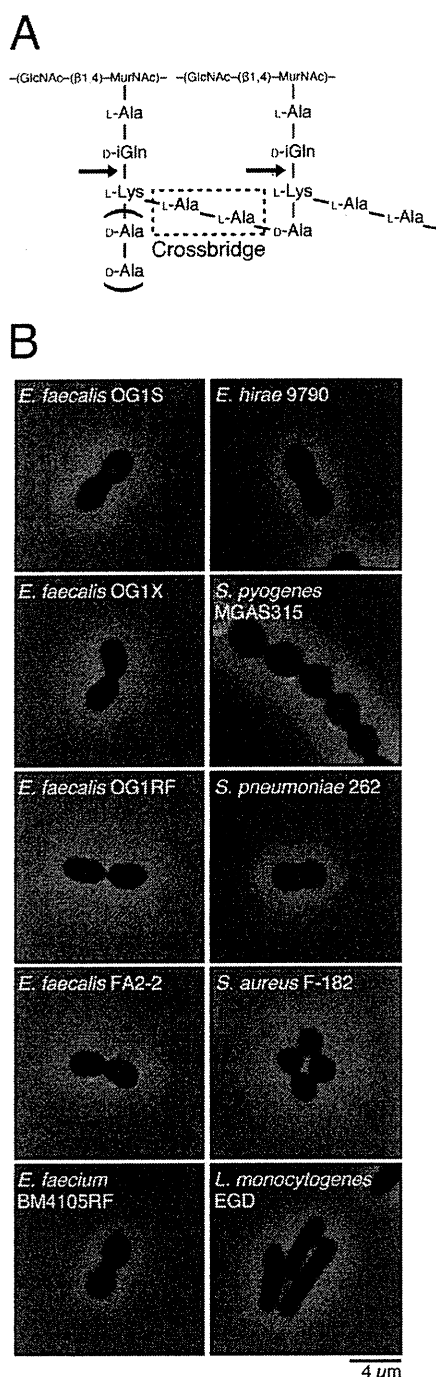

FIG 6 $BacL_1$ localization in various Gram-positive bacterial species. (A) Peptidoglycan structure of *E. faecalis*, representing an example of the organization of peptide chain-cross-linking by a dipeptide. The dotted-line frame indicates the cross-bridging peptide between stem peptides bound to *N*-acetylmuramic acids. Arrows indicate the sites of cleavage by the endopeptidase activity of $BacL_1$. (B) Overnight cultures of Gram-positive bacteria, diluted 5-fold with fresh THB broth, were treated with HiLyte Fluor 555-labeled (red) $BacL_1$ (5 μg/ml). After incubation for 1 h, the cells were fixed and analyzed by fluorescence microscopy. Phase contrast is pseudocolored (green) in the merged images.

composed of L-Ala-LAla-cross-bridged peptidoglycan that is likely to be recognized by $BacL_1$, there must be an additional determinant(s) for the localized targeting of $BacL_1$ to the cell division-associated sites. The equatorial ring is a characteristic structure observed at the middle of ovococcus cells (32, 51, 52). This ring structure marks the initiation site for the peripheral cell wall-synthesizing machinery to construct the new peptidoglycan during cell elongation. Therefore, $BacL_1$ might recognize the cell wall-synthesizing machinery complex that is formed at the equatorial ring or division septum during cell division. Alternatively, the relatively extended distribution of $BacL_1$, from equatorial ring to division septum, raises the possibility that $BacL_1$ preferentially binds to newly synthesized nascent cell wall. Martínez et al. demonstrated that a bacteriocin of *Lactococcus lactis*, lactococcin 972, inhibits the septum formation to cause abnormal cell morphology in sensitive target cells. Although they have not shown this, lactococcin 972 itself might be associated with the cell-dividing structure, like $BacL_1$ (53). Understanding the determinant(s) restricting the targeting site of $BacL_1$ to cell division-related areas requires further analysis.

As shown by the results in Fig. 3, the SH3 repeat moiety of $BacL_1$ was required and sufficient for its localized targeting. These repeats are present in the region from amino acid 329 to 590 of $BacL_1$ (see Fig. S2A in the supplemental material). These individual SH3 repeats are nearly identical to each other (see Fig. S2B). The SH3 domain sequences of $BacL_1$ also show significant homology to SH3 domains from other bactericidal proteins (see Fig. S2C), such as ALE-1 from *S. aureus* (54). Crystal structure analysis of the SH3 domain in ALE-1 revealed that the N-terminal conserved motif YXXXNKYGTXYXXXESA is a recognition groove that specifically binds to penta-Gly-cross-bridging peptides in *S. aureus* peptidoglycan (42). The YXXXNKYGTXYXXXESA motif (see Fig. S2C, blue frames) is not present in the SH3 domain of $BacL_1$. Instead, extra conserved residues (see Fig. S2C, red frames) are present among the SH3 domains targeting bacteria with an L-Ala-L-Ala-cross-bridged cell wall, including *E. faecalis*, *Streptococcus agalactiae*, and *S. pneumoniae*. Furthermore, amino acids 15 and 14 in the N terminus and C terminus, respectively, are highly conserved motifs (see Fig. S2C, magenta highlighting) among the three SH3 domains of $BacL_1$, suggesting that these conserved motifs in $BacL_1$ may play a role in the specific recognition of the L-Ala-L-Ala-cross-bridged peptidoglycan structure.

Lysostaphin, with activity specific against *S. aureus*, is able to distinguish the penta-Gly-cross-bridging structure in the peptidoglycan of *S. aureus* from the cross-bridging structures of other peptidoglycans (55). The lysostaphin-specific immunity factor Lif, a FemABX-like protein, incorporates serine into the cross-bridging peptides in peptidoglycan of *S. aureus* and converts it from the penta-Gly-type cross bridge (56). This conversion of the cross-bridging peptide in peptidoglycan results in resistance to lysostaphin. Zoocin A is a bacteriolytic endopeptidase against the cell wall of sensitive bacteria produced by *Streptococcus equi* subsp. *zooepidemicus* strain 4881 (57). The cross bridge in peptidoglycan of *S. equi* is an L-Ala-L-Ala peptide and is susceptible to the peptidoglycan hydrolase activity of zoocin A (57). Zif, an immunity factor of zoocin A, belongs to the FemABX-like protein family (58). It additively increases L-Ala residues in the cross bridges of peptidoglycans and converts L-Ala-L-Ala into L-Ala-L-Ala-L-Ala, resulting in resistance to zoocin A activity. Meanwhile, BacI,

TABLE 2 Summary of cross-bridge structure and phenotypes against Bac41 in various bacterial species

			Presence of phenotype[a]	
Species	Strain	Cross-bridging peptide	Targeting of BacL$_1$[b]	Susceptibility to Bac41[c]
Enterococcus faecalis	OG1S	L-Ala-L-Ala	+	+
Enterococcus faecalis	OG1X	L-Ala-L-Ala	+	+
Enterococcus faecalis	OG1RF	L-Ala-L-Ala	+	+
Enterococcus faecalis	FA2-2	L-Ala-L-Ala	+	±
Enterococcus faecium	BM4105RF	L-Asp	−	−
Enterococcus hirae	9790	D-Asn	−	−
Streptococcus pyogenes	MGAS315	L-Ala-L-Ala	+	−
Streptococcus pneumoniae	262	L-Ala-L-Ala/L-Ser	±	−
Staphylococcus aureus	F-182	Gly_5	−	−
Listeria monocytogenes	EGD	NA[d]	−	−

[a] +, clear/positive; ±, obscure/weak; −, negative.
[b] Targeting of BacL$_1$ was determined from the results shown in Fig. 6B.
[c] Susceptibility to Bac41 (BacL$_1$ and BacA mixture) was determined by a soft-agar-based bacteriocin assay.
[d] NA, not applicable; *L. monocytogenes* has direct bridging between stem peptides.

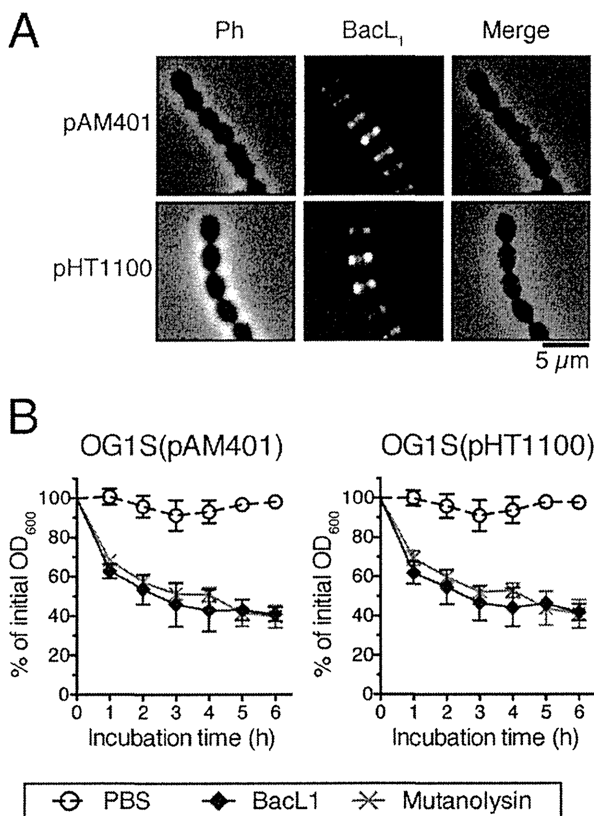

FIG 7 Involvement of Bac41 specific immunity factor BacI in the susceptibility of cell wall to BacL$_1$. (A) An overnight culture of *E. faecalis* carrying pAM401 (a vector control without the *bacI* gene) or pHT1100 (a pAM401 derivative containing all Bac41 genes, including immunity factor *bacI*) diluted 5-fold with fresh THB broth was treated with HiLyte Fluor 555-labeled (red) BacL$_1$ (5 μg/ml). After incubation for 1 h, the cells were fixed and analyzed by fluorescence microscopy. Phase contrast (Ph) is pseudocolored (green) in merged images. (B) A cell wall fraction prepared from *E. faecalis* carrying pAM401 or pHT1100 in exponential phase was diluted with PBS. Recombinant BacL$_1$ (5 μg/ml) or mutanolysin (1 μg/ml) was added to the cell wall suspension, and the mixture incubated at 37°C. The turbidity at 600 nm was quantified at the indicated times during incubation. The values presented are the percentages of the initial turbidity of the respective samples. The PBS-treated sample is presented in each graph as a negative control. The data are presented as the mean results ± SD of three independent experiments.

which is the cognate immunity factor against Bac41, did not affect the BacL$_1$ targeting (Fig. 7A). In addition, the cell wall fraction prepared from *E. faecalis* that is resistant to Bac41 due to the presence of *bacI* was still susceptible to the peptidoglycan-degrading activity of BacL$_1$ (Fig. 7B), suggesting that BacI is not involved in the activity of BacL$_1$. This result suggests the possibility that BacI confers immunity by acting on the function of BacA rather than that of BacL$_1$ or that another factor(s) of target cells, such as molecules or receptors that are only present in the growing cells, is involved in the BacI-mediated resistance.

The bactericidal activity of Bac41 (BacL$_1$ and BacA) is strictly specific against *E. faecalis*, and Bac41 does not show any activity against the other bacterial species tested (Table 2). The specificity could be partially explained by the diversity of cross-bridging peptides of peptidoglycan among bacterial species. As demonstrated by the results in Fig. 6B, BacL$_1$ appears to discriminate target bacterial species from nontarget species by specific recognition of L-Ala-L-Ala-cross-bridged peptidoglycan. Indeed, BacL$_1$ is able to target bacteria with L-Ala-L-Ala-cross-bridged peptidoglycan, such as *S. pyogenes* and *S. pneumoniae*, regardless of the bacterial genus. In contrast, *E. faecium* and *E. hirae*, with peptidoglycans cross bridged by L-Asp and D-Asn, respectively, were not recognized by BacL$_1$ although they are phylogenetically classified in the same genus as *E. faecalis*. These observations demonstrated that the activity of BacL$_1$ is specific against bacteria with L-Ala-L-Ala-cross-bridged peptidoglycans. However, the bacteriolytic phenotype in the copresence of BacL$_1$ and BacA appears to be more complex (Table 2). The bactericidal effect of BacL$_1$ and BacA (Bac41) was observed only against *E. faecalis* even though other bacteria are of the L-Ala-L-Ala-cross-bridge type. Interestingly, *S. pyogenes* and *S. pneumoniae* were not susceptible to BacL$_1$ and BacA although they were targeted with BacL$_1$. One possibility is that BacA is not able to access *S. pyogenes* and *S. pneumoniae*. Furthermore, the susceptibility of *E. faecalis* FA2-2 to BacL$_1$ and BacA was lower than that of *E. faecalis* OG1-derived strains, such as OG1S, OG1X, and OG1RF. Thurlow et al. reported that enterococcal capsular polysaccharide is present in FA2-2 but not in OG1 strains (59). Thus, probably the capsule on the cell surface of strain FA2-2 cells limits the access of BacA, resulting in the decreased susceptibility to Bac41-induced lysis. To reveal the detailed mo-

lecular mechanism of the Bac41 module, further functional analysis of BacA is needed.

The Bac41-mediated fratricide module excludes *E. faecalis* strains without the Bac41-encoding plasmid. Therefore, this module is inferred to play a role in the effective expansion of the Bac41-carrying plasmid. Our conclusion that cell growth is required for cell lysis by $BacL_1$ and BacA (Fig. 4 and 5) is consistent with the hypothesis because selection is involved in possible plasmid loss during distribution to daughter cells. Hence, it is reasonable that the Bac41 system works only when bacteria are allowed to grow, replicate DNA, and distribute plasmid to daughter cells. Our results in this study suggest a novel player involved in the plasmid maintenance system.

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Identification of a Second Two-Component Signal Transduction System That Controls Fosfomycin Tolerance and Glycerol-3-Phosphate Uptake

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Particular interest in fosfomycin has resurfaced because it is a highly beneficial antibiotic for the treatment of refractory infectious diseases caused by pathogens that are resistant to other commonly used antibiotics. The biological cost to cells of resistance to fosfomycin because of chromosomal mutation is high. We previously found that a bacterial two-component system, CpxAR, induces fosfomycin tolerance in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7. This mechanism does not rely on irreversible genetic modification and allows EHEC to relieve the fitness burden that results from fosfomycin resistance in the absence of fosfomycin. Here we show that another two-component system, TorSRT, which was originally characterized as a regulatory system for anaerobic respiration utilizing trimethylamine-*N*-oxide (TMAO), also induces fosfomycin tolerance. Activation of the Tor regulatory pathway by overexpression of *torR*, which encodes the response regulator, or addition of TMAO increased fosfomycin tolerance in EHEC. We also show that phosphorylated TorR directly represses the expression of *glpT*, a gene that encodes a symporter of fosfomycin and glycerol-3-phosphate, and activation of the TorR protein results in the reduced uptake of fosfomycin by cells. However, cells in which the Tor pathway was activated had an impaired growth phenotype when cultured with glycerol-3-phosphate as a carbon substrate. These observations suggest that the TorSRT pathway is the second two-component system to reversibly control fosfomycin tolerance and glycerol-3-phosphate uptake in EHEC, and this may be beneficial for bacteria by alleviating the biological cost. We expect that this mechanism could be a potential target to enhance the utility of fosfomycin as chemotherapy against multidrug-resistant pathogens.

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Although fosfomycin is classified as an old antibiotic, it was recently revived as an antibiotic that could be effective against multidrug-resistant (MDR) pathogens, such as extended-spectrum-β-lactamase (ESBL) producers (1). This antibiotic has no structural relationship to other commonly used antibiotics; therefore, it is not affected by the development of cross-resistance in MDR pathogens (2, 3). Fosfomycin is also used to decrease the risk of development of hemolytic-uremic syndrome (HUS), which is a fatal infectious disease caused by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (4–6).

Fosfomycin is an antagonist of phosphoenolpyruvate (PEP) and inhibits MurA activity, which transfers PEP to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine in the initial step of bacterial cell wall biosynthesis (7). GlpT, which is a glycerol-3-phosphate transporter, and UhpT, which is a glucose-6-phosphate transporter, are involved in the uptake of fosfomycin (8–11). Mutations in the genes encoding these proteins that result in impaired fosfomycin binding or uptake confer resistance to fosfomycin. In some *E. coli* studies, mutations in genes encoding the positive regulators of *uhpT* expression, UhpA and CyaA, confer resistance because these mutants have a reduced uptake of fosfomycin (12, 13). Other studies showed that a clinical isolate that is resistant to fosfomycin produces a MurA variant that results in the overexpression of MurA (14).

However, these mutations cause irreversible defects in GlpT, UhpT, and/or MurA production and thus affect GlpT- and UhpT-dependent carbon substrate uptake and MurA-catalyzed cell wall synthesis, which impose a fitness burden on the cells. Mutants that are resistant to fosfomycin can frequently be isolated *in vitro* in rich media (15, 16), while epidemiologic data indicate that susceptibility rates have remained relatively stable, despite the prevalent use of fosfomycin (17–19). Thus, the fitness cost of fosfomycin resistance may account for the relatively low level of fosfomycin resistance seen in the clinical setting. However, the Clinical and Laboratory Standards Institute (CLSI) reported that the *in vivo* antibacterial activity of fosfomycin against *E. coli* species is relatively lower than that of other commonly used antibiotics, such as β-lactams and fluoroquinolones (20). We asked if there is an innate mechanism of tolerance to this drug that does not rely on genetic modifications, as this could serve as a potential target to enhance the utility of fosfomycin.

Previously, we found that the CpxAR pathway, a two-component system (TCS), in EHEC induces fosfomycin tolerance (21).

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype[a]	Reference or source
Strains		
HH-H7-008	Parent strain (EHEC O157:H7 strain RIMD 0509952 containing *tnaA* and *lacZI* deletions)	27
HH-H7-040	*cpxA* mutant from HH-H7-008	21
HH-H7-093	*cpxA cpxR* double mutant from HH-H7-008	21
HH-H7-095	*glpT* mutant from HH-H7-008	This work
HH-H7-121	*torR* mutant from HH-H7-008	This work
HH-H7-122	*torS* mutant from HH-H7-008	This work
HH-H7-123	*torT* mutant from HH-H7-008	This work
HH-H7-157	*cpxA torR* double mutant from HH-H7-008	This work
MG1655	*E. coli* K-12 wild-type reporter strain	28
Rosetta(DE3)	T7 expression strain, Cm^r	Novagen/EMD Bioscience
CFT073	Uropathogenic *E. coli* strain	29
Plasmids		
pKO3	Temperature-sensitive vector for gene targeting, *sacB* Cm^r	30
pTrc99A	Vector for IPTG-inducible expression, Ap^r	31
pTrc99torR	TorR overexpression plasmid, Ap^r	This work
pQE80	Vector for expression of His-tagged protein, Ap^r	Qiagen
pQE80torR	N-terminal His$_6$-TorR overexpression plasmid, Ap^r	This work
pNN387	Single-copy plasmid with promoterless *lacZ*, Cm^r	32
pNNglpT-P	*glpT* promoter reporter, Cm^r	This work
pNNglpT-P1	pNNglpT-P with deletion of the *glpT* promoter in the TorR-binding site, Cm^r	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

The CpxAR pathway is activated in the presence of sub-MICs of fosfomycin, and then a transient decrease in GlpT- and UhpT-dependent transport confers fosfomycin tolerance and reduces the fitness cost associated with it. The reversible mechanism, which does not rely on genetic mutations to confer fosfomycin tolerance, may be a beneficial strategy for bacteria to alleviate the fitness burden conferred by fosfomycin resistance in fosfomycin-free circumstances.

EHEC has ~30 pairs of proteins composing the TCS, including CpxAR (22). We are interested in the relationship between these TCS proteins and the reversible control of fosfomycin tolerance. In addition to CpxAR, we found that another TCS, the Tor system, controls fosfomycin tolerance. The Tor system regulates the utilization of trimethylamine-*N*-oxide (TMAO) as an electron acceptor for enterobacteria, including EHEC, during anaerobic respiration (23). TorS is the sensor kinase, and it is autophosphorylated by sensing the complex of TMAO and a periplasm-binding protein, TorT. TorS then transfers the phosphate to its cognate response regulator, TorR (24, 25). The phosphorylated TorR activates a subset of genes that encode proteins involving the TMAO reductase system (26).

In this study, we found that TorSRT coordinates the reversible control of fosfomycin tolerance. The TorR protein activated by the TMAO signal repressed the *glpT* gene encoding a glycerol-3-phosphate/fosfomycin cotransporter. The TorR-activated cells had reduced fosfomycin uptake, resulting in fosfomycin tolerance. However, the fosfomycin-tolerant cells had a decreased capability for glycerol-3-phosphate uptake, suggesting a trade-off between fosfomycin tolerance and glycerol-3-phosphate utilization.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, all bacteria were grown in Luria-Bertani (LB) medium. To anaerobically grow EHEC strains, we used Hungate tubes (Bello Glass Inc., Vineland, NJ) equipped with a rubber stopper and a screw cap. For marker selection and plasmid maintenance, antibiotics were added to the growth medium at the following concentrations: 150 μg/ml ampicillin and 15 μg/ml chloramphenicol. For growth experiments using carbon source-limited medium, EHEC strains were grown in LB medium at 37°C with shaking for 12 to 16 h. The pellets were washed twice with glucose-free Dulbecco's modified Eagle medium (DMEM) and resuspended in half of the original culture volume with the same medium. The cell suspensions were diluted into fresh medium supplemented with 0.5% glycerol-3-phosphate or glucose at a 1:100 ratio. The bacteria were grown at 37°C with shaking, and cell growth was monitored by determination of the absorbance at 660 nm.

Cloning and mutant constructions. In-frame deletions of *torR*, *torS*, and *torT* were constructed by sequence overlap extension PCR according to a strategy described previously (30) with primer pairs torR-delta1/torR-delta2 and torR-delta3/torR-delta4 for *torR*, primer pairs torS-delta1/torS-delta2 and torS-delta3/torS-delta4 for *torS*, and primer pairs torT-delta1/torT-delta2 and torT-delta3/torT-delta4 for *torT* (Table 2). The upstream flanking DNA included 450 bp and the first 4 amino acid codons for *torR*, the first 5 amino acid codons for *torS*, and the first 4 amino acid codons for *torT*. The downstream flanking DNA included the last 10 amino acid codons for *torR*, the last 3 amino acid codons for *torS*, the last 10 amino acid codons for *torT*, the stop codon, and 450 bp of DNA. These deletion constructs were ligated into BamHI- and SalI-digested temperature-sensitive vector pKO3 and introduced into HH-H7-008, the parent strain (27). We selected sucrose-resistant, chloramphenicol-sensitive colonies at 30°C and confirmed the resulting mutant strains using PCR analysis and DNA sequencing. We also constructed a mutant with a deletion of the *glpT* gene by the same method using primer pairs glpT-delta1/glpT-delta2 and glpT-delta3/glpT-delta4.

To construct *torR* expression plasmid pTrc99torR, the *torR* gene was amplified with the primer pair pTrc-torR-F/pTrc-torR-R (Table 2). The product was digested with NcoI and BamHI and ligated into similarly digested plasmid pTrc99A. The His$_6$-TorR expression plasmid pQE80-torR was constructed by ligating the *torR* gene that had been PCR amplified with primers pQE-torR-F and pQE-torR-R into BamHI- and Hin-

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