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Original Article

Clinical Implications of Biofilm Formation by *Enterococcus faecalis* in the Urinary Tract

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The potential relationships between biofilm formation and pathogenicity of *Enterococcus faecalis* in urinary tract infections (UTI) were investigated. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated UTI (one isolate per patient) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2'')*). The production of biofilm, hemolysin and gelatinase by these isolates was also examined and the associated medical records of patients were retrospectively reviewed. Of 352 *E. faecalis* isolates, 315 possessed *asa1* and/or *esp* genes. Of the 63 hemolysin- and 167 gelatinase-producing isolates, 59 and 94 isolates, respectively, possessed both *asa1* and *esp* genes. *E. faecalis* isolates with both *asa1* and *esp* genes formed biofilms at significantly higher rates than those with neither gene ($P = 0.038$). The genes encoding *asa1*, *cylA* and *aac(6')/aph(2'')* were transferable and appeared to have accumulated in these isolates. The *E. faecalis* isolates possessing *asa1* and/or *esp* genes were found from both catheter-related or -unrelated UTI. Our study indicates that *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

Key words: *Enterococcus faecalis*, urinary tract infection, biofilm formation, pathogenicity, gene transfer

Enterococcus faecalis is a normal commensal in the human intestinal flora but can lead to nosocomial infections [1-5]. Although the pathogenicity of *E. faecalis* in the urinary tract is not considered high, *E. faecalis* has been isolated from the urinary tract at increasing frequencies: up to 20% of urinary isolates in some reports [6-8]. Several virulence factors have been described in *E. faecalis*, including aggregation substance (Agg), enterococcal surface protein (Esp), cytolysin (Cyl) having both hemolytic and bactericidal activity, and gelatinase (Gel) [1, 3, 4]. These factors have been thought to act synergistically to enhance virulence by facilitating achievement of a quorum and activating the quorum-sensing mode of regulation, resulting in tissue damage and potentially deeper tissue invasion [1, 9-12]. Recent studies have shown that enterococci form biofilms and that the *E. faecalis fsr* quorum-sensing system controls biofilm development [13-17].

Agg is a surface protein expressed by the *asa1* gene, which is located on pheromone-responsive *E. faecalis* plasmids [18, 19]. It is produced in response to pheromones secreted by potential recipient *E. faecalis* cells,

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and causes the aggregation of donor and recipient cells, thereby facilitating the transfer of plasmids that may carry virulence traits and antibiotic resistance genes [18, 19]. Agg may also play an important role in the onset of enterococcal infection by facilitating the adherence of enterococci to cardiac vegetations as well as to the epithelial cells of the intestine, kidney and urinary tract [1]. Esp encoded by the chromosomal *esp* is associated with increased virulence, colonization and persistence in the urinary tract, along with biofilm formation [1, 13, 17, 20, 21]. The production of Cyl has also been shown to significantly worsen the severity of endocarditis and endophthalmitis in animal models as well as to contribute to the severity of enterococcal disease in humans [1, 22]. Cyl is either encoded within pheromone-responsive plasmids or on the chromosome within pathogenicity islands and is transcribed as an operon that contains at least 5 genes including *cylA* [1, 22]. Gel, encoded by the chromosomal *gelE*, is an extracellular zinc-metalloprotease that hydrolyzes collagen, gelatin, and small peptides and that has been shown to exacerbate endocarditis in an animal model [1].

In the present study, we investigated the potential relationships between biofilm formation and clinical implications of *E. faecalis* isolates in the urinary tract. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated urinary tract infection (UTI) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2")*). In addition, the production by these isolates of biofilm and extracellular enzymes, hemolysin (Hln) and Gel was examined, and the associated medical records of the patients were retrospectively reviewed. The data were summarized in 4 groups based on the presence/absence of *asa1* and *esp* genes encoding enterococcal adhesins, Agg and Esp, respectively.

Materials and Methods

Bacterial isolates from patients with UTI. The *E. faecalis* bacterial isolates used in this study were isolated from patients with complicated UTI at the Department of Urology, Okayama University Hospital, over a 12-year period from 1991 through 2002. A total of 352 isolates that grew to $\geq 10^4$ CFU/ml in urinary culture were selected for this study. All 352 patients (one isolate per patient) had documented pyuria (WBC ≥ 5/hpf).

Polymerase chain reaction (PCR) assay. The presence of *asa1*, *esp*, *cylA* *gelE/sprE* and *aac(6')/aph(2")*, which encode Agg, Esp, Cyl, Gel/serine protease and gentamicin resistance, respectively, was confirmed by PCR assay. The primers and PCR conditions used in this study are summarized in Table 1. Multiplex PCR assay was used to detect the *asa1* and *cylA* genes using primers reported by Huycke *et al.* [23]. Primers reported by Shankar *et al.* [20] were used for amplification within the N-terminal region of *esp*. Primers reported by Nakayama *et al.* [24] and Van de Klundert *et al.* [25] were used to amplify *gelE/sprE* and *aac(6')/aph(2")* genes, respectively. DNA amplification was carried out by the method of Kariyama

Table 1 PCR primers and conditions used in this study

Primer specificity	Primer sequences	Product length [bp]	PCR conditions				Reference
			Initial denaturation	Cycling	Cycle	Final extention	
asa1	F: 5'-GATTCTTCGATTGTGTTGTAAACG-3' R: 5'-GGTGCCACAATCAAATTAGG-3'	380	2 min, 95 °C	1 min, 95 °C; 1 min, 46 °C; 1 min, 72 °C	35	10 min, 72 °C	23
esp	F: 5'-TTGCTAATGCTAGTCCACGACC-3' R: 5'-GCGTCAACACTTGCATTGCCGAA-3'	955	2 min, 95 °C	45 sec, 94 °C; 45 sec, 63 °C; 2 min, 72 °C	30	7 min, 72 °C	20
cylA	F: 5'-GGGGATTGATAGGCTTCATCC-3' R: 5'-GCACCGACGGTAATTACAGACTCTAG TCCTCC-3'	432	2 min, 95 °C	1 min, 95 °C; 1 min, 46 °C; 1 min, 72 °C	35	10 min, 72 °C	23
gelE/sprE	F: 5'-ATGAAGGGAAATAAAATTTTATAC-3' R: 5'-CTGCTGGCACAGCGGATA-3'	2428	2 min, 94 °C	30 sec, 94 °C; 30 sec, 48 °C; 3 min, 72 °C	35	6 min, 72 °C	24
aac(6')/aph(2")	F: 5'-CCAAGAGCAATAAGGGCATA-3' R: 5'-CACTATCATAACCACTACCG-3'	220	5 min, 94 °C	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	35	10 min, 72 °C	25

et al. [26]. Briefly, total cellular DNA was prepared as follows: 0.5 ml of *E. faecalis* culture, grown overnight in Todd Hewitt broth (Becton Dickinson and Company, Sparks, MD, USA), was centrifuged, and the pellet was resuspended in 50 μl of InstaGene (Bio-Rad Laboratories, Hercules, CA, USA). After the suspension was heated for 10 min at 100 °C, 2.5 μl of the supernatant was mixed with 22.5 μl of prepared reaction mixture to start the reaction. The primer pairs were added to the respective reaction mixtures. The 25-μl reaction volume contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of *Taq* DNA polymerase (Takara Shuzo, Shiga, Japan). PCR products were analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, gels were stained with ethidium bromide (1 mg/l) and photographed under a UV trans-illuminator. A 100-bp DNA Ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker. The fragment sizes of PCR products are shown in Table 1.

Detection of hemolysin-producing isolate. Production of hemolysin was determined by plating *E. faecalis* isolates onto Todd Hewitt agar plates supplemented with 5% rabbit blood and incubated at 37 °C for 48 h. When hemolysis was observed on the plate at 48 h, the isolate was considered a hemolysin-producing isolate.

Detection of gelatinase-producing isolate. Production of gelatinase was determined by the method of Su *et al*. [27]. A transparent halo around colonies after exposure to a solution saturated with ammonium sulfate on the surface of the medium was considered a gelatinase-positive response.

Biofilm formation assay. *E. faecalis* isolates were grown overnight at 37 °C in tryptic soy broth supplemented with 0.25% glucose [15]. The culture was diluted 1:100 in medium, and 200 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 24 h at 37 °C without shaking, wells were gently washed three times with 300 μl of distilled water, dried in an inverted position, and stained with 300 μl of 2% crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 300 μl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells.

Conjugative transfer experiments. Mating experiments were performed by the method of Clewell *et al*. [28]. Each of 43 *E. faecalis* isolates possessing the 3 genes *asa1*, *cylA* and *aac(6')/aph(2")* was used as donor, and *E. faecalis* FA2-2 (rifampicin and fusidic acid resistance) was used as recipient. Broth matings were performed with a donor/recipient ratio of 1:10. Overnight cultures of 0.05 ml of donor and 0.5 ml of recipient were added to 4.5 ml of fresh broth, and the mixtures were incubated at 37 °C with gentle agitation for 4 h. Portions (0.1 ml) of the mixed and diluted culture were then plated on Todd Hewitt agar plates with appropriate selective antibiotics. Transconjugants were selected on Todd Hewitt agar plates supplemented with 500 μg of gentamicin/ml and 25 μg of rifampicin and fusidic acid/ml. Colonies were counted after 48 h of incubation at 37 °C. Separate platings where donors alone were selected provided a basis for estimating the transfer frequency (per donor). Filter matings were also carried out. Overnight cultures of 0.1 ml of donor and 1 ml of recipient were added to 9 ml of fresh broth, and the mixtures were immediately collected on a membrane (25 mm width 0.45 μm pore size filter, type HA; Millipore Corp., Billerica, MA, USA), which was inverted onto the surface of a Todd Hewitt agar plate supplemented with 4% horse blood. After incubation at 37 °C for 20 h, the cells were suspended in 1 ml of Todd Hewitt broth. The subsequent procedure was the same as that for broth matings. Transfer frequencies were calculated as the number of transconjugants per donor cell.

Retrospective clinical study. We retrospectively reviewed the medical records of the 352 patients whose characteristics were summarized in Table 2 and classified their UTI as catheter-related or catheter-unrelated, polymicrobial or monomicrobial, and febrile or non-febrile cases. Febrile UTI was defined as UTI in a patient with a body temperature of $\geq$ 37.0 °C.

Statistical methods. Data are expressed as mean values $\pm$ standard deviation (SD). Comparison of OD_{570} values between groups was carried out using

Table 2 Demographic and clinical characteristics of 352 patients with UTI due to *E. faecalis*

Characteristics	Value
Age; median ± SD (range)	61 ± 20.7 (0–94)
Sex; no. male/no. female	240/112
Polymicrobial infections	257 isolates
Pseudomonas aeruginosa	42
Escherichia coli	28
Methicillin-resistant *Staphylococcus aureus*	22
Klebsiella pneumoniae	19
Serratia marcescens	17
Staphylococcus epidermidis	13
Citrobacter freundii	13
Staphylococcus aureus	9
Candida albicans	7
Proteus mirabilis	6
Others	81
Underlying diseases	
Bladder cancer	90
Prostatic cancer	49
Other urinary tract cancer	10
Neurogenic bladder	79
Benign prostatic hyperplasia	40
Urinary tract stones	19
Ureteral stricture	7
Ureteropelvic junction stenosis	6
Vesicoureteral reflux	5
Others	47

Fisher's exact test or Mann-Whitney's U test. All results were considered statistically significant at the $P < 0.05$ level.

Results

Presence of various genes, and the production of extracellular enzymes in E. faecalis isolates. Of the 352 *E. faecalis* isolates, 291 (82.7%), 254 (72.2%), 164 (46.6%), 306 (86.9%), and 141 (40.1%) isolates possessed *asa1, esp, cylA, gelE/sprE,* and *aac(6')/aph(2")*, respectively. Of 164 isolates possessing the *cylA* gene, 63 (38.4%) isolates produced Hln. Of 306 isolates possessing *gelE/sprE* genes, 167 (54.6%) isolates produced Gel. The number of *E. faecalis* isolates with both *asa1* and *esp* genes, with *asa1* gene only, with *esp* gene only, and with neither gene were 230, 61, 24 and 37, respectively.

Biofilm formation of E. faecalis isolates. Of the 352 *E. faecalis* isolates, 64 (18.2%), 156 (44.3%), and 132 (37.5%) isolates exhibited strong ($OD_{570} \geq 0.5$), medium ($OD_{570} \geq 0.2$ to < 0.5), and weak (OD_{570} 0 to < 0.2) biofilm formation, respectively. The mean OD_{570} of the 352 isolates was 0.36 ± 0.37 (mean ± SD). We evaluated the relationships between biofilm formation and the 4 virulence determinants of the *E. faecalis* isolates. As shown in Table 3, the mean OD_{570} value (mean ± SD) was significantly higher in *asa1*-, *esp*-, and *cylA*-positive isolates than in *asa1*-, *esp*-, and *cylA*-negative isolates ($P = 0.0176$, $P = 0.0276$ and $P = 0.0116$, respectively). The value was also significantly higher in Hln producing isolates than in Hln non-producing isolates ($P = 0.0384$). We also evaluated the biofilm-forming capacities of *E. faecalis* isolates in the 4 groups based on the presence/absence of *asa1* and *esp* genes (Fig. 1). As shown with a box and whisker plot, the *E. faecalis* isolates with *asa1* and *esp* genes had greater capacities for biofilm formation than did those lacking these genes. The Mann-Whitney's U test of 2 mean OD_{570} values (mean ± SD), 0.41 ± 0.42 and 0.22 ± 0.16, in 230 *asa1*-, *esp*- positive and 37 *asa1*-, *esp*-negative isolates, respectively, confirmed that the *E. faecalis* isolates possessing both *asa1* and *esp* genes had significantly greater capacities for biofilm formation than did those lacking both genes ($P = 0.038$).

Percentage of E. faecalis isolates possessing cylA, gelE/sprE and aac(6')/aph(2'') genes in 4 groups based on the presence/absence of asa1 and esp genes. As shown in Fig. 2, the percentage of *E. faecalis* isolates possessing *cylA* and/or *aac(6')/aph(2")* genes was greatest in the group with both *asa1* and *esp* genes and lower in the groups with only the *asa1* gene or the *esp* gene. No isolates possessing the *cylA* gene and only 1 isolate possessing the *aac(6')/aph(2")* gene were found in the group lacking both the *asa1* and *esp* genes. In contrast, *E. faecalis* isolates possessing *gelE/sprE* genes were found evenly among the 4 groups. Of the 230 *E. faecalis* isolates in the group with *asa1* and *esp* genes, 147 (63.9%), 195 (84.8%) and 117 (50.9%) possessed *cylA, gelE/sprE* and *aac(6')/aph(2")* genes, respectively. Of the 61 *E. faecalis* isolates in the group with only the *asa1* gene, 15 (24.6%), 56 (91.8%) and 22 (36.1%) possessed the *cylA, gelE/sprE, and aac(6')/aph(2")*genes, respectively. Of the 24 *E. faecalis* isolates in the group with only the *esp* gene, 2 (8.3%), 23 (95.8%) and 1 (4.2%) possessed the *cylA, gelE/sprE,* and *aac(6')/aph(2")* genes, respectively. Of the 37 *E. faecalis*

Table 3 Relationship between biofilm-forming capacities and virulence factors/clinical background

	Number of isolates	OD_{570} (mean ± SD)	*P* value (Mann-Whitney's *U* test)
Total isolates tested	352	0.36 ± 0.37	
Virulence determinants			
asa1-positive	291	0.38 ± 0.38	0.0176
asa1-negative	61	0.27 ± 0.27	
esp-positive	254	0.40 ± 0.41	0.0276
esp-negative	98	0.26 ± 0.18	
cylA-positive	164	0.41 ± 0.41	0.0116
cylA-negative	188	0.32 ± 0.32	
gelE/sprE-positive	306	0.36 ± 0.35	0.0915
gelE/sprE-negative	46	0.35 ± 0.46	
Extracellular enzymes			
hemolysin producing	63	0.47 ± 0.48	0.0384
hemolysin non-producing	289	0.34 ± 0.33	
gelatinase producing	167	0.35 ± 0.31	0.1376
gelatinase non-producing	185	0.37 ± 0.42	
Clinical background			
catheter-related	107	0.33 ± 0.34	0.0582
catheter-unrelated	245	0.38 ± 0.38	
polymicrobial	202	0.35 ± 0.34	0.5505
monomicrobial	150	0.37 ± 0.40	
febrile	60	0.31 ± 0.32	0.1267
non-febrile	292	0.37 ± 0.38	

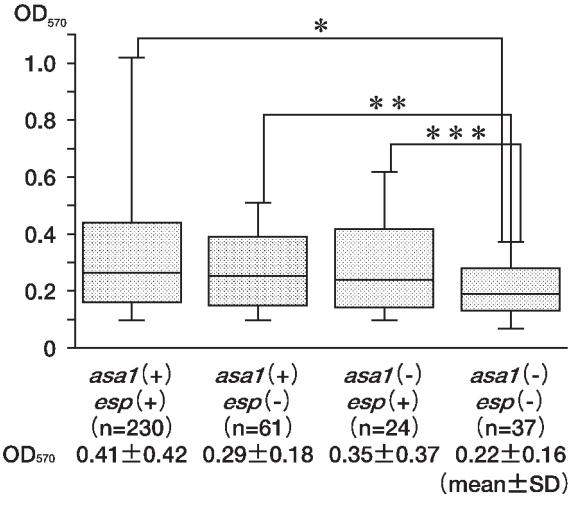

Fig. 1 Biofilm-forming capacities of *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes. OD_{570} values of the isolates in the 4 groups are shown by the box and whiskers plot, which represents a five-number summary (upper extreme, upper quartile, median, lower quartile, and lower extreme). The mean OD_{570} values (mean ± SD) of the 4 groups are also shown.
*$P = 0.038$; **$P = 0.0449$; ***$P = 0.1208$ (Mann-Whitney's *U* test).

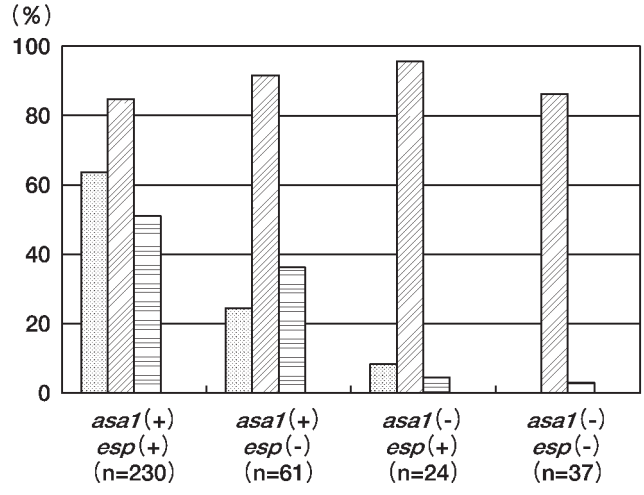

Fig. 2 Percentage of *cylA*-, *gelE/sprE*- and *aac(6')/aph(2'')*-positive isolates among *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes.
Bars: ⁘, *cylA*; ▨, *gelE-sprE*; ▤, *aac(6')-aph(2'')*

isolates in the group with neither gene, 0 (0%), 32 (86.5%) and 1 (2.7%) possessed the *cylA*, *gelE/sprE*, and *aac(6')/aph(2")* genes, respectively.

Number of E. faecalis isolates producing hemolysin and gelatinase in 4 groups based on the presence/absence of asa1 and esp genes. As shown in Fig. 3, the majority of Hln-producing and Gel-producing isolates were found in the group with both *asa1* and *esp* genes. Of the 63 Hln-producing and 167 Gel-producing isolates, 59 (93.7%) and 94 (56.3%) isolates, respectively, possessed both the *asa1* and *esp* genes.

Transferability of asa1, cylA and aac(6')/aph(2'') genes. To determine the transferability of *asa1*, *cylA* and *aac(6')/aph(2")* genes, mating experiments were performed. Of the 43 *E. faecalis* isolates possessing the 3 genes of *asa1*, *cylA* and *aac(6')/aph(2")*, 4 isolates were able to donate gentamicin resistance at a frequency of 10^{-4} to 10^{-1} per donor in broth matings. In filter matings, 7 of 43 and 28 of 43 isolates were able to donate the gentamicin resistance at a frequency of 10^{-4} to 10^{-1} and 10^{-8} to 10^{-5} per donor, respectively. The presence of *asa1*, *cylA* and *aac(6')/aph(2")* genes in transconjugants was confirmed by PCR assay. Of the transconjugants from the 35 *E. faecalis* isolates possessing *asa1*, *cylA* and *aac(6')/aph(2")*, 35 (100%) possessed the *aac(6')/aph(2")* gene, 33 (94.3%) possessed the *asa1* gene and 12 (34.3%) possessed the *cylA* gene.

Clinical aspects on the isolation of E. faecalis in 4 groups based on the presence/absence of asa1 and esp genes. The 352 cases of UTI caused by *E. faecalis* consisted of 107 catheter-related (30.4%) and 245 catheter-unrelated (69.6%) cases, 202 polymicrobial (57.4%) and 150 monomicrobial (42.6%) cases, and 60 febrile (17.0%) and 292 non-febrile (83.0%) cases. No statistically significant differences between biofilm-forming capacities and clinical background (catheter-related and catheter-unrelated cases, polymicrobial and monomicrobial cases, febrile and non-febrile cases) were found (Table 3). As shown in Fig. 4, both *asa1* and *esp* genes were carried by 20, 46, 79 and 85 isolates from patients with catheter-related monomicrobial UTI, catheter-related polymicrobial UTI, catheter-unrelated monomicrobial UTI and catheter-unrelated polymicrobial UTI, respectively. The *asa1* and/or *esp* genes were carried on 80 of 82 (97.6%) and 103 of 124 (83.1%) isolates from patients with catheter-related

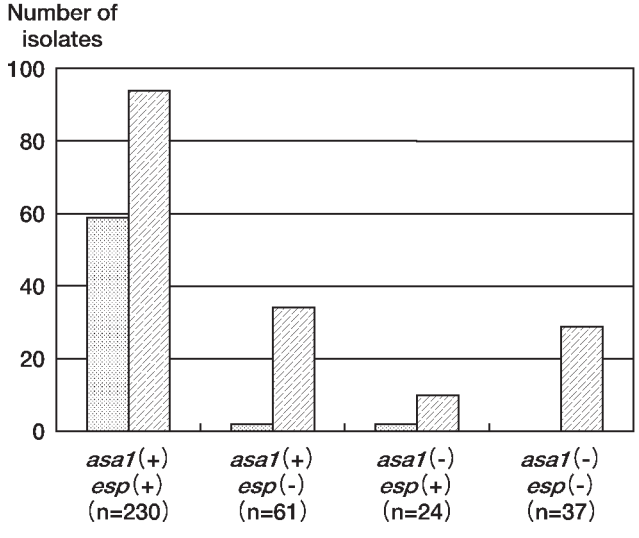

Fig. 3 Number of *E. faecalis* isolates producing hemolysin and gelatinase in 4 groups based on the presence/absence of *asa1* and *esp* genes.
Bars: ⁘, hemolysin; ▨, gelatinase

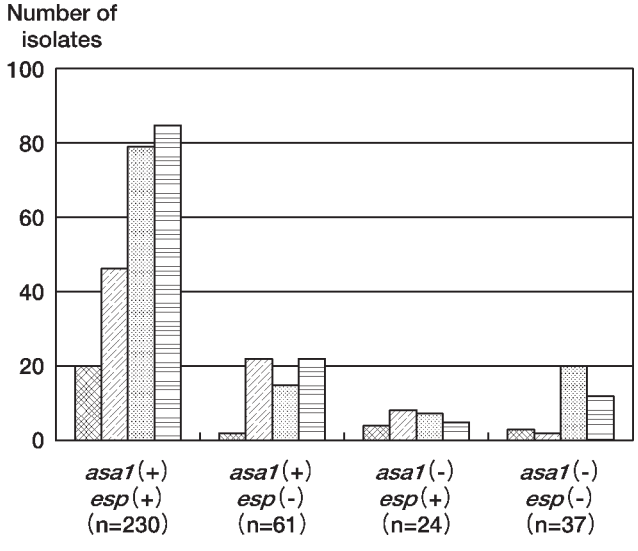

Fig. 4 Number of *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes. Clinical aspects are shown by 4 categories.
Bars: ▩, catheter-related monomicrobial UTI
▨, catheter-related polymicrobial UTI
⁘, catheter-unrelated monomicrobial UTI
▤, catheter-unrelated polymicrobial UTI

polymicrobial UTI and catheter-unrelated monomicrobial UTI, respectively, (Fisher's exact test: $P = 0.0020$).

Discussion

Enterococci are an important cause of nosocomial infections [3-5]. Although *E. faecalis* in the urinary tract rarely causes serious infectious symptoms, the frequency of isolation of *E. faecalis* from the urinary tract of hospitalized patients has risen [6-8]. One of the reasons, we suspect, is that the number of patients with various urinary stents and catheters is increasing with the progress of endourology in the urology ward. Therefore, it is important to understand biofilm formation and the pathogenicity of *E. faecalis* infections in the urinary tract. Biofilms are surface-associated, sessile bacterial communities. A mature biofilm is formed when planktonic cells initially colonize a surface, aggregate and/or grow into multicellular colonies, and embed themselves in an exopolysaccharide matrix [29, 30]. Enterococci have been associated with biofilms on various kinds of indwelling medical devices [29]. An understanding of the bacterial factors that foster enterococci in the nosocomial environment or at infection sites is only recently emerging.

The incidence of virulence factors in *E. faecalis* clinical isolates has been studied [31-33]. In 1995, Coque *et al*. [31] reported that frequencies of Hln, Gel and *asa1* in *E. faecalis* urine isolates were 13, 53 and 67 %, respectively. In 2002, Vergis *et al*. [32] reported that frequencies of Hln, Gel and *esp* in *E. faecalis* blood isolates were 11, 64 and 32%, respectively. In our study, frequencies of Hln, Gel, *asa1* and *esp* in *E. faecalis* urine isolates were 63/352 (17.9%), 167/352 (47.4%), 291/352 (82.7%) and 254/352 (72.2%), respectively. Our data indicated that *E. faecalis* isolates possessing both *asa1* and *esp* were predominant. As shown in Fig. 2, *E. faecalis* isolates possessing *asa1* were more likely to contain *cylA* and/or *aac(6')/aph(2")*. This may be due to the expression of *asa1*-encoded Agg facilitating the subsequent exchange of genetic material between *E. faecalis* isolates. The additional presence of virulence factors may enhance the ability of pathogenic *E. faecalis* to persist in the clinical environment.

Horizontal gene transfer is important for the evolution and genetic diversity of natural microbial communities [34]. The prevalence of plasmids in bacteria from diverse habitats is well established, and gene transfer by conjugation is one of the best understood mechanisms for dissemination of genetic information. Since most bacteria in natural settings reside within biofilms, it follows that conjugation is a likely mechanism by which bacteria in biofilms transfer genes within or between populations. In this study, we chose *E. faecalis* isolates possessing the 3 genes *asa1*, *cylA* and *aac(6')/aph(2")* to examine gene transfer from one *Enterococcus* to another. These 3 genes have been reported to be encoded on pheromone-responsive *E. faecalis* plasmids [23, 35]. Our data indeed demonstrated the existence of highly conjugative virulence genes and antimicrobial resistance genes in *E. faecalis* isolates from patients with UTI.

With regard to biofilm formation, there were contrasting reports on the role of Esp and Gel. A strong correlation between the presence of Esp and the ability of an enterococcal strain to form biofilms *in vitro* has been reported [13]. In the same study, however, it was suggested that additional determinants in *E. faecalis* may also contribute to biofilm formation. More recently, Kristich *et al*. [14] demonstrated that an *esp*-negative strain can form biofilms on abiotic surfaces independently of Esp. Mohamed *et al*. [15] also demonstrated that *esp* was not required to form biofilm, but that its presence was associated with higher amounts of biofilm. In the same study, several genes of *E. faecalis* that influenced primary attachment and biofilm formation (*epa*, *atn*, *gelE*, and *fsr*) were identified. Most recently, Tendolkar *et al*. [17] defined Esp as a key contributor to the ability of *E. faecalis* to form biofilms in a glucose-dependent manner. In addition, Kristich *et al*. [14] reported that Gel enhanced biofilm formation by *E. faecalis*, whereas Tendolkar *et al*. [17] did not find a synergistic effect between Gel and Esp on biofilm formation. In our study, biofilm-forming capacities were significantly higher in *esp*-positive isolates than in *esp*-negative isolates (Table 3). On the other hand, there were no significant differences between *gelE/sprE*-positive, Gel producing isolates and *gelE/sprE*-negative, Gel non-producing isolates on biofilm-forming capacities (Table 3).

In our study, *cylA*-positive, Hln producing *E. faecalis* isolates formed biofilms at rates significantly higher than those of *cylA*-negative, Hln non-producing isolates ($P = 0.0116$ and $P = 0.0384$, respectively). To our knowledge, there has been no report on Cyl of *E. faecalis* implicating it in biofilm formation. Caiazza *et al*. [36] showed that Hla, a 34-kDa protein of *Staphylococcus aureus* that causes host cell lysis by heptamerizing upon insertion into eukaryotic cell membranes, plays a

primary role in cell-to-cell interactions during biofilm formation. They were initially surprised to find that a secreted toxin had such a dramatic impact on biofilm formation. More recently, we showed that the biofilm-forming capacities of MRSA isolates were higher in *hla*- and *hlb*-positive isolates than in *hla*- and *hlb*-negative isolates, respectively [37]. These toxins may be bifunctional enzymes and cause tissue damage of urinary epithelium.

The *fsr* quorum-sensing system has been shown to regulate 2 proteases, Gel and serine proteases [1, 10–12]. More recently, Hancock *et al.* [16] showed that the *E. faecalis fsr* quorum-sensing system controls biofilm development through the production of Gel. However, our data do not support this finding since Gel non-producing isolates can form biofilms (Table 3). In our previous study [24], a 23.9-kilobase chromosomal deletion containing the *fsr* gene cluster region was found to be present in the majority of Gel non-producing isolates. An understanding of the process of biofilm formation by *E. faecalis* is only now beginning to emerge, and the results appear to be contradictory [38].

In this study, there were no statistically significant differences between biofilm-forming capacities and clinical background (catheter-related and catheter-unrelated cases, polymicrobial and monomicrobial cases, febrile and non-febrile cases). Biofilm formation by enterococci occurs not only with indwelling devices but also in response to any bacterial factor that mediates adherence to components of the extracellular matrix of the host [1, 39, 40]. No single factor predominated as the major predictor of virulence, and their effects appeared to be cumulative [37]. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown. Host factors for *E. faecalis* disease are likely to include a genetic predisposition via one or more susceptibility genes and acquired factors such as the presence of intravenous devices, surgical wounds, and other events that perturb normal host defenses.

In summary, our study indicates that *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

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