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 ≥ 37.0°C.

Statistical methods. Data are expressed as mean values ± standard deviation

(SD). Comparison of OD_{570} values between groups was carried out using 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} ≥0.5), medium (OD_{570} ≥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 four 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 four 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 isolates 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 four 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* 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 four

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 four 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 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 three 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 two 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.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research (C) (2003-2004, No. 15591688) from the Japan Society for the Promotion of Science and by a Grant-in-Aid from the Ministry of Health, Labour and Welfare (H16-medical treatment-014).

References

1. Gilmore MS, Coburn PS, Nallapareddy SR and Murray BE: Enterococcal virulence. In: Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE and Rice LB eds, The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington DC (2002) pp 301-354.
2. Hancock LE and Gilmore MS: Pathogenicity of enterococci. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA and Rood JI eds, Gram-positive pathogens. American Society for Microbiology, Washington DC (2000) pp 251-258.
3. Mundy LM, Sahm DF and Gilmore MS: Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev (2000) 13: 513-522.
4. Huycke MM, Sahm DF and Gilmore MS: Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis (1998) 4: 239-249.

5. Murray BE: Diversity among multidrug-resistant enterococci. Emerg Infect Dis (1998) 4: 37-47.
6. Ronald A: The etiology of urinary tract infection: traditional and emerging pathogens. Am J Med (2002) 113: 14S-19S.
7. National Nosocomial Infections Surveillance System. National nosocomial infections surveillance (NNIS) system report, data summary from January 1990-May 1999, issued June 1999. Am J Infect Control (1999) 27: 520-532.
8. Gatermann SG: Virulence factors of *Staphylococcus saprophyticus*, *Staphylococcus epidermidis,* and enterococci. In: Mobley HLT and Warren JW eds, Urinary tract infections: molecular pathogenesis and clinical management. American Society for Microbiology, Washington DC (1996) pp 313-340.
9. Haas W, Shepard BD and Gilmore MS: Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature (2002) 415: 84-87.
10. Qin X, Singh KV, Weinstock GM and Murray BE: Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect Immun (2000) 68: 2579-2586.
11. Nakayama J, Cao Y, Horii T, Sakuda S, Akkermans ADL, de Vos WM and Nagasawa H: Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. Mol Microbiol (2001) 41: 145-154.

12. Qin X, Singh KV, Weinstock GM and Murray BE: Characterization of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. J Bacteriol (2001) 183: 3372-3382.

13. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva J, Penadés JR and Lasa I: The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilim formation. Appl Environ Microbiol (2001) 67: 4538-4545.

14. Kristich CJ, Li Y-H, Cvitkovitch DG and Dunny GM: Esp-independent biofilm formation by *Enterococcus faecalis*. J Bacteriol (2004) 186: 154-163.

15. Mohamed JA, Huang W, Nallapareddy SR, Teng F and Murray BE: Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. Infect Immun (2004) 72: 3658-3663.

16. Hancock LE and Perego M: The *Enterococcus faecalis fsr* two-component system controls biofilm development through production of gelatinase. J Bacteriol (2004) 186: 5629-5639.

17. Tendolkar PM, Baghdayan AS, Gilmore MS and Shankar N: Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. Infect Immun (2004) 72: 6032-6039.

18. Clewell DB: Sex pheromone systems in enterococci. In Dunny GM and Winans SC eds, Cell-cell signaling in bacteria. American Society for Microbiology, Washington DC (1999) pp47-65.

19. Clewell DB and Dunny GM: Conjugation and genetic exchange. In: Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE and Rice LB eds, The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington DC (2002) pp265-300.
20. Shankar V, Baghdayan AS, Huycke MM, Lindahl G and Gilmore MS: Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infect Immun (1999) 67: 193-200.
21. Shankar N, Lockatell CV, Baghdayan AS, Drachenberg C, Gilmore MS and Johnson DE: Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect Immun (2001) 69: 4366-4372.
22. Coburn PS and Gilmore MS: The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. Cell Microbiol (2003) 5:661-669.
23. Huycke MM and Gilmore MS: Frequency of aggregation substance and cytolysin genes among enterococcal endocarditis isolates. Plasmid (1995) 34: 152-156.
24. Nakayama J, Kariyama R and Kumon H: Description of a 23.9-kilobase chromosomal deletion containing a region encoding *fsr* genes which mainly determines the gelatinase-negative phenotype of clinical isolates of *Enterococcus faecalis* in urine. Appl Environ Microbiol (2002) 68: 3152-3155.
25. Van de Klundert JAM and Vliegenthart JS: PCR detection of genes coding for aminoglycoside-modifying enzymes. In: Persing DH, Smith TF, Tenover FC and White TJ eds, Diagnostic molecular microbiology: principles and applications.

American Society for Microbiology, Washington DC (1993) pp547-552.

26. Kariyama R, Mitsuhata R, Chow JW, Clewell DB and Kumon H: Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol (2000) 38: 3092-3095.
27. Su YA, Sulavik MC, He P, Makinen KK, Makinen PL, Fiedler S, Wirth R and Clewell DB: Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. *liquefaciens*. Infect Immun (1991) 59: 415-420.
28. Clewell DB, An FY, White BA and Gawron-Burke C: *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn*918*). J Bacteriol (1985) 162: 1212-1220.
29. Donlan RM and Costerton JW: Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev (2002) 15: 167-193.
30. Hall-Stoodley L, Costerton JW and Stoodley P: Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol (2004) 2: 95-108.
31. Coque TM, Patterson JE, Steckelberg JM and Murray BE: Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J Infect Dis (1995) 171: 1223-1229.
32. Vergis EN, Shankar N, Chow JW, Hayden MK, Snydman DR, Zervos MJ, Linden PK, Wagener MM and Muder RR: Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface

protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. Clin Infect Dis (2002) 35: 570-575.

33. Waar K, Muscholl-Silberhorn AB, Willems RJL, Slooff MJH, Harmsen HJM and Degener JE: Genogrouping and incidence of virulence factors of *Enterococcus faecalis* in liver transplant patients differ from blood culture and fecal isolates. J Infect Dis (2002) 185: 1121-1127.
34. Davey ME and O'toole GA: Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev (2000) 64:847-867.
35. Patterson JE and Zervos MJ: High-level gentamicin resistance in *Enterococcus*: microbiology, genetic basis, and epidemiology. Rev Infect Dis (1990) 12: 644-652.
36. Caiazza NC and O'Toole GA: Alpha-toxin is required for biofilm formation by *Staphylococcus aureus*. J Bacteriol (2003) 185: 3214-3217.
37. Ando E, Monden K, Mitsuhata R, Kariyama R and Kumon H: Biofilm formation among methicillin-resistant *Staphylococcus aureus* isolates from patients with urinary tract infection. Acta Med Okayama (2004) 58: 207-214.
38. Carniol K and Gilmore MS: Signal transduction, quorum-sensing, and extracellular protease activity in *Enterococcus faecalis* biofilm formation. J Bacteriol (2004) 186: 8161-8163.
39. Rozdzinski E, Marre R, Susa M, Wirth R and Muscholl-Silberhorn A: Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilized extracellular matrix proteins. Microb Pathog (2001) 30: 211-220.

40. Tomita H and Ike Y: Tissue-specific Adherent *Enterococcus faecalis* strains that show highly efficient adhesion to human bladder carcinoma T24 cells also adhere to extracellular matrix proteins. Infect Immun (2004) 72: 5877-5885.

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℃	1 min, 95℃; 1 min, 46℃; 1 min, 72℃	35	10 min, 72℃	23
esp	F: 5'-TTGCTAATGCTAGTCCACGACC-3' R: 5'-GCGTCAACACTTGCATTGCCGAA-3'	955	2 min, 95℃	45 sec, 94℃; 45 sec, 63℃; 2 min, 72℃	30	7 min, 72℃	20
cylA	F: 5'-GGGGATTGATAGGCTTCATCC-3' R: 5'-GCACCGACGGTAATTACAGACTCTAGTCCTCC-3'	432	2 min, 95℃	1 min, 95℃; 1 min, 46℃; 1 min, 72℃	35	10 min, 72℃	23
gelE/sprE	F: 5'-ATGAAGGGAAATAAAATTTTATAC-3' R: 5'-CTGCTGGCACAGCGGATA-3'	2428	2 min, 94℃	30 sec, 94℃; 30 sec, 48℃; 3 min, 72℃	35	6 min, 72℃	24
aac(6')/aph(2")	F: 5'-CCAAGAGCAATAAGGGCATA-3' R: 5'-CACTATCATAACCACTACCG-3'	220	5 min, 94℃	1 min, 94℃; 1 min, 55℃; 1 min, 72℃	35	10 min, 72℃	25

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

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	

Legends to Figures

Fig. 1 Biofilm-forming capacities of *E. faecalis* isolates in four groups based on the presence/absence of *asa1* and *esp* genes. OD_{570} values of the isolates in the four 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 four 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 four groups based on the presence/absence of *asa1* and *esp* genes.

Bars: ▩ , *cylA* ; ▨ , *gelE-sprE* ; □ , *aac(6')-aph(2")*

Fig. 3 Number of *E. faecalis* isolates producing hemolysin and gelatinase in four groups based on the presence/absence of *asa1* and *esp* genes.

Bars: ▩ , hemolysin ; ▨ , gelatinase

Fig. 4 Number of *E. faecalis* isolates in four groups based on the presence/absence of *asa1* and *esp* genes. Clinical aspects are shown by four categories.

Bars: ▩ , catheter-related monomicrobial UTI

▨ , catheter-related polymicrobial UTI

▩ , catheter-unrelated monomicrobial UTI

▤ , catheter-unrelated polymicrobial UTI

Fig.1