Pathogenesis of nosocomial infections with Enterococcus faecalis

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Pathogenesis of *Enterococcus faecalis* Infections
Review of the Literature

CHAPTER 1
Introduction

Enterococci are Gram positive coccoid bacteria that belong to normal microbiota of the gastrointestinal tract of humans, most mammals, birds, and many other species. In the colon of nearly all humans enterococci can be found in numbers as high as $10^8$ colony-forming units per gram of feces (164, 182). From the more than 13 enterococcal species that are described, *Enterococcus faecalis* and *Enterococcus faecium* are isolated most frequently. In the normal healthy host enterococci seldom are causing infections, only some urinary tract infections are seen. However, surveillance data indicate that enterococci are becoming one of the leading causes of nosocomial infections (142, 159, 204). Nosocomial infections are infections that patients acquire in a health-care institution. These infections can be caused by transmission of the bacterium from patient to patient or from the health care worker to the patient. Nosocomial infections with enterococci are frequently seen in critically ill patients at intensive care units, for example in liver transplant patients, which are often considered especially vulnerable to enterococcal infections (248, 250). The problem of nosocomial enterococcal infection is compounded by emerging antibiotic resistance (113). Different studies describe a longer length of stay in hospital and increased mortality due to vancomycin-resistant *E. faecium* compared to vancomycin-susceptible *E. faecium* (70, 140). However, resistance alone does not explain the increase of enterococci in nosocomial infections. Although resistance is relatively uncommon among *E. faecalis* isolates compared to resistance among *E. faecium* isolates (113), *E. faecalis* currently accounts for the majority of clinical enterococcal isolates (up to 90 %), followed by *E. faecium* (142, 206). This disparity might be explained by the relative abundance of *E. faecalis* in the gastrointestinal tract (164, 182) or enhanced virulence of *E. faecalis*. This report will focus on *E. faecalis* infections as these are more prominent among hospital acquired infections.

The pathogenesis of enterococcal infections is quite complex because under most circumstances, enterococci stably coexist with the host and are often even considered beneficial to the host. However, situations can arise where this balanced commensalism is disrupted. This disruption may result from a breakdown in host mechanisms that hold commensal organisms in control. For example, the natural barrier of the skin or mucosa might be disrupted in hospitalized patients that underwent large operations in the abdominal cavity or received indwelling biomaterial implants like drains or catheters. An increasing number of patients also receives immune suppressive therapy e.g. after solid organ transplantation, suppression of the immune response can also lead to infection with commensal microorganisms. Disruption may further result from the organism acquiring new traits that enable the organism to circumvent the host defense or to adhere to biomaterial implants and cause biomaterial associated infections. The goal of this introduction is to highlight different aspects of the pathogenesis and clinical manifestation of (nosocomial) *E. faecalis* infection, both from the side of the host as well the side of the microorganism.
Virulence of *E. faecalis*

Enterococcal virulence factors can contribute to enterococcal disease in different ways; by enhancing colonization, adherence and invasion of host tissues, by modulation of the host immunity, and by inducing pathological changes in the host associated with increased severity of infection (123, 125, 167). Generally, the pathogenesis of enterococcal infections follows a common sequence of events and different virulence factors of *E. faecalis* can play a role in each of these steps (95, 167). First, enterococci possessing various virulent traits asymptomatically colonize the gastro-intestinal tract (233, 229). Subsequently, this population is expanded and in some patients the enterococci can invade the host tissue and cause disease. Factors that enhance the virulence of enterococci through this way are found more frequently among isolates from hospitalized patients compared to isolates from the community. Finally, enterococci can cause symptomatic disease at the level of tissue destruction or toxicity (39, 117, 213, 223, 226). The virulence factors enhance pathogenicity through this way would not necessarily appear in increased numbers among various clinical isolates but would be associated with more severe clinical presentation. Therefore, the contribution of certain enterococcal traits to virulence can be proven by a higher incidence in nosocomial isolates and/or increased severity of disease in patients or animals.

The contribution of virulence factors to the pathogenesis of nosocomial infection is somewhat blurred due to the fact that patients with high immune suppression are also susceptible to commensal strains without virulence factors (9, 50, 93). Therefore, collections of infection-derived hospital isolates possibly contain a spectrum of types of strains, from pure commensals to strains harboring a combination of virulence factors. Strains that cause hospital ward outbreaks among different groups of patients might represent the true virulent enterococcal lineages; therefore the ability to cause outbreaks should be included in the analysis of a possible virulence trait. Below the different stages of pathogenesis and the role of different virulence factors herein are discussed.

**Colonization, adherence and invasion of host tissues**

Clinical studies have shown that nosocomial enterococcal infections often arise through transfer of the enterococci between patients (7, 37, 114, 131, 172, 240, 247). Patients often get colonized with nosocomial enterococci previous to the development of an enterococcal infection (95, 275). One of the questions of nosocomial enterococcal infection is why these nosocomial enterococci so easily colonize niches that are already occupied by members of the same species. These nosocomial enterococci might have extra capabilities to colonize, overgrow and invade host tissues. These capabilities might be supported by the use of antibiotics without significant anti-enterococcal activity, thus outcompeting the indigenous flora and creating niches which can be readily colonized by the nosocomial enterococci (20, 273). This is supported by the fact that many nosocomial enterococci are more resistant to antibiotics compared to commensal enterococci (113, 214). Amplified fragment length polymorphism (AFLP) analysis of *E. faecium* isolates from different clinical subgroups and particular hosts indicated that these isolates could be divided into
genogroups with an AFLP similarity of > 65%. Interestingly, a clear dichotomy between isolates from hospitalized and non-hospitalized persons was detected (261). This association of a specific genogroup with a particular host or environment might hold the key to the question why nosocomial enterococci colonize the hospitalized patient and its environment more easily.

Studies in mice showed that following colonization of the GI tract, bacteria may be able to translocate over the epithelial lining of the lumen (225). The proposed mechanism for the translocation of intestinal bacteria is that epithelial cells and phagocytes uptake the gastrointestinal bacteria and the bacteria exit at the apical side or migrate in phagocytes to the mesenteric lymph nodes, proliferate and spread to distant sites (14, 258). Evidence of translocation of \textit{E. faecalis} across the intact intestinal epithelium was given by Wells et al. who cultured \textit{E. faecalis} from liver, spleen and lymph nodes of mice with intestinal overgrowth of \textit{E. faecalis} (255, 257). Furthermore, they showed that these translocated enterococci caused systemic infections (225). In another study, Wells et al. suggested that the site of translocation in mice was the caecum or colon (254).

In an in vitro study Guzman et al. provided evidence for the role of adherence in the pathogenesis of \textit{E. faecalis} urinary tract infection and endocarditis (101, 102). They showed that isolates from urinary tract infections or endocarditis adhered best to respectively urinary tract epithelial cells and heart cells and that these adhesive properties were induced by growth in serum (102).

**Modulation of the host immunity**

\textit{E. faecalis} must overcome the clearance functions of the host system to successfully cause infection. Polymorphonuclear leukocytes (PMNs) are a critical component of the human host response against bacterial infections. Invading bacteria may be coated by complement proteins or specific antibodies and subsequently phagocytosed and killed by PMNs. This process of coating of bacteria with complement proteins or antibodies to enhance phagocytosis is called opsonization. Studies involving the role of antibodies and complement in the phagocytic killing of enterococci revealed that PMN mediated killing depended primarily on complement activation by either the classical or the alternative pathway (5, 106). Antibodies to \textit{E. faecalis} enhanced the PMN mediated killing, however they were not essential as different studies showed efficient killing also in the presence of serum without gamma globulins (84, 106).

Although antibodies to enterococci are found in humans with enterococcal infections (229), studies on the efficacy of antibodies to \textit{E. faecalis} in the prevention of infections are quite contradicting. Huebner et al. (110) found prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide in a mouse infective model. In addition, the role of antibodies to the surface protein aggregation substance (Agg) in prevention of endocarditis is underscored by the absence of host antibodies specific for the Agg during the formation of endocardial vegetation. Thereby the bacteria are protected from the influence of the antibodies (155). However, another study to the efficacy of antibodies to Agg in the prevention of endocarditis in a rabbit model did not show any protection (154).

\textit{E. faecalis} has developed different strategies to overcome the immune response. Gentry-
Weeks et al. (92) reported a prolonged intracellular survival of enterococci for up to 72 h in mouse peritoneal macrophages. This property might contribute to the pathogenesis of infections in the way that the enterococci migrate to distant sites in the body and be protected from antimicrobial therapy within the macrophage. In line with these findings are the results of other investigations reporting that Agg promotes direct, opsonin-independent binding of \textit{E. faecalis} to PMN and that through this opsonin-independent binding \textit{E. faecalis} was able to survive inside different phagocytes (201, 231, 246). Another study showed that strains expressing gelatinase, cytolysin, or Agg were not more resistant to neutrophil-mediated killing, but the in vitro assays were performed under circumstances that might not support expression of these traits or mimic the in vivo situation (5, 184). The structure of the enterococcal surface protein (Esp) with multiple repeat motifs in the encoding gene might be important in the immune evasion of infecting \textit{E. faecalis} (217).

**Pathological changes in the host**

The last step in the pathogenesis of infections is the production of pathologic changes in the host. Such changes can be induced by the host inflammatory response or by direct tissue damage as a result of secreted toxins or proteases. Enterococcal lipoteichoic acid is most frequently described as one of the factors that modulates the host immune response and thereby causes tissue damage. Several groups found lipoteichoic acid to be as inflammatory as lipopolysaccharide of Gram-negative bacteria and a potent inducer of different cytokines (17, 238). A study to the role of Agg and enterococcal binding substance to the pathogenesis of endocarditis found that strains without Agg or enterococcal binding substance lacked the ability to cause disease, strains with either Agg or enterococcal binding substance were intermediate virulent and strains with both Agg and enterococcal binding substance on their surface exhibited the greatest ability to cause disease. Furthermore, none of the rabbits receiving Agg and enterococcal binding substance positive organisms showed gross pericardial inflammation. The lethality and lack of inflammation are consistent with the presence of a superantigen (213). Secreted products of \textit{E. faecalis} that can cause direct tissue damage are cytolysin and gelatinase (123). The effects of these toxins will be discussed below.

**Virulence traits of \textit{E. faecalis}**

Several possible virulence traits are described in literature, including different enterococcal adhesins that are expressed on the surface of \textit{E. faecalis} like Agg or Esp (95). These adhesins possibly play a role in the adhesion to different biological surfaces like epithelial cells and synthetic biomaterial surfaces like indwelling catheters. Furthermore, some virulence factors are described that are excreted into the environment like cytolysin and gelatinase (95). These factors might play a role in the toxicity of the enterococcus and the survival of the microorganism in different environments. In Table 1 the results of different studies reporting the incidence of virulence factors in \textit{E. faecalis} isolates from different sources are summarized. The results of the different studies are quite variable, depending on the sources of the \textit{E. faecalis} isolates and the methods of determination of the virulence factors. By adding up all results of the different studies for medical and commensal isolates, a
significant difference between the two groups can be found for the cytolysin and the gelatinase trait and the presence of the esp gene. Comparing the different studies this way might be controversial as different methods of detection were used. Recent studies showed an incongruence between the genotypic and phenotypic detection of cytolysin, gelatinase and Agg with lower percentages in the phenotypic detection (46, 69). This may be due to the presence of silent genes that are not expressed under the test conditions. Below the different virulence factors and their possible contribution to the pathogenesis of infections are summarized. A schematic representation of most virulence factors and their working mechanism is given in figure 1.

Aggregation substance and sex-pheromone plasmid system
The Agg was first described as part of the sex-pheromone system, a very efficient system through which E. faecalis can collect plasmids (63, 65). Figure 2 gives a schematic overview

Figure 1. Schematic representation of the involvement of the different virulence factors in pathogenesis.
of the transfer mechanism of this plasmid collecting system (41, 64, 265). The conjugation involves three steps: (i) the excretion of small linear peptides, the sex-pheromones, by strains not possessing the corresponding sex-pheromone plasmid, (ii) the sex-pheromone induces a response in donor cells carrying the corresponding sex-pheromone plasmid, these cells start to express a plasmid-encoded surface protein, the aggregation substance, (iii) the aggregation substance enables close cell-cell contact between donor and recipient and transfer of the plasmid. Many different sex-pheromone plasmids encoding different virulence traits and antibiotic resistances are described (265). DNA hybridization studies have shown that all different sex-pheromone plasmids contain a homologous DNA region that encodes the Agg (e.g. Asa1 encoded on pAD1), except the Agg on plasmid pAM373 (Asa373), which does not fit the overall homology (50, 88, 175). Sequencing of the asa1 gene encoding Agg showed that this adhesin contained two RGD (arg/gly/asp) motifs that are also present e.g. in fibronectin in which it mediates the binding to integrin receptors present on eukaryotic
cells (87, 208). To date, the sex-pheromone system has been confined to \textit{E. faecalis} and was not observed in other enterococcal species although the transfer of plasmid through this system is an interesting object of study as this would explain transfer of antibiotic resistance and virulence genes. The only evidence for interaction with other bacterial species is the fact that expression of Asa373 is also induced by a pheromone produced by \textit{Staphylococcus aureus} (42, 176).

The finding of the RGD motif in Asa1 led to the investigation of the in vitro adherence of \textit{E. faecalis} expressing Agg to eukaryotic cells. Kreft et al. (132) found a sevenfold higher adhesion to renal tubular cells of \textit{E. faecalis} constitutively expressing Agg compared to a plasmid free control strain. Interestingly, the binding capacity could be reduced by the addition of RGD peptide to the epithelial cells prior to the binding assay thus blocking the receptors on the epithelial cells. Further studies report the role of Agg in the invasion of enterococci in intestinal epithelial cells or colonic mucosa and show that the presence of Agg on the surfaces promotes the internalization of enterococci into these tissues to levels similar to that of \textit{Salmonella typhimurium} (119, 185), however Agg did not increase the adhesion and internalization to cells derived from the ileum (212). Another argument for the role of Agg in interaction with epithelial cells was the fact that Agg expression in \textit{Lactococcus lactis} also increased the internalization to cultured colonic enterocytes (259). Waters et al. (252) used mutations constructed in Asc10, the Agg encoded by the plasmid pCF10, to prove that the Asc10 functional domain and not the RGD motif was critical for efficient internalization by enterocytes. This is in contrary to earlier reports about the role or the RGD motif in the interaction with epithelial cells. They also found that expression of Asc10 in the non-aggregating \textit{E. faecalis} strain INY3000 did not mediate the adhesion which indicated that apart from the Asc10 functional domain other enterococcal traits might play a role in the eukaryotic cell internalization. This might also explain the difference with earlier reports on the role of the RGD motif in internalization.

Table 1 shows that Asa1 is present in about 50 % of both medical and commensal isolates, providing evidence that although it might enhance host bacterium interaction, it is not a prerequisite for infection. A reason for the lack of enrichment for Asa1 among medical isolates may be the variable expression of Asa1 in vivo. Agg is produced upon induction by its homologous pheromone. However, serum factors might also induce expression of Agg (108, 132). Only few studies investigated the presence of Asa373 in \textit{E. faecalis} isolates, these studies showed that the general incidence is much lower than the incidence of Asa1. The role of Agg in pathogenesis was further studied in different animal models. However, results were not consistent and depended on the strains and models used. Two studies to the role of Agg in endocarditis in rabbits found that Agg contributed to the development of endocarditis. However, additional factors, cytolysin or binding substance, were necessary to develop serious disease (39, 213). Another animal study with rats failed to find a contribution of Agg to the development of endocarditis (15), as well as an experimental study to the development of endophthalmitis in rabbits (122) and a study to the virulence of \textit{E. faecalis} in the roundworm \textit{Caenorhabditis elegans} (91).

In conclusion, the Agg might contribute to the pathogenesis of \textit{E. faecalis} infections at different levels. First, it plays a role in the dissemination of plasmids encoding virulence factors or antibiotic resistance. Furthermore, Agg plays a role in the immune evasion of \textit{E.}}
## Table 1. Prevalence of virulence factors in *E. faecalis* isolates from various sources and the exclusion of clonal relationship as reported in the literature.

<table>
<thead>
<tr>
<th>material</th>
<th>no. of isolates</th>
<th>hemolysin</th>
<th>gelatinase</th>
<th>aggregation substance</th>
<th>reference</th>
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<td>Asa373</td>
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<td>Esp</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>reference</td>
<td></td>
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<tr>
<td>various medical samples</td>
<td>97</td>
<td>58 (60)</td>
<td>48 (49)(^e)</td>
<td>68 (70)(^c)</td>
<td>not excluded</td>
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<tr>
<td>feces medical students</td>
<td>23</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>190</td>
<td>85 (45)(^p)</td>
<td></td>
<td></td>
<td>not excluded</td>
</tr>
<tr>
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<td>118</td>
<td>24 (20)(^c)</td>
<td></td>
<td></td>
<td>not excluded</td>
</tr>
<tr>
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<td>35</td>
<td>4 (11)</td>
<td>14 (40)</td>
<td></td>
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<td>blood</td>
<td>39</td>
<td>6 (16)</td>
<td>12 (32)</td>
<td></td>
<td>PFGE</td>
</tr>
<tr>
<td>feces healthy volunteers</td>
<td>14</td>
<td>0(^d)</td>
<td>9 (64)(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>endocarditis</td>
<td>44</td>
<td>7 (16)</td>
<td>24 (54)</td>
<td>23 (52)</td>
<td>excluded with</td>
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<td>various medical samples</td>
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<td>32 (37)</td>
<td>50 (58)</td>
<td>62 (72)</td>
<td>PFGE</td>
</tr>
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<td>feces hospital patients</td>
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<td>10 (31)</td>
<td>20 (62)</td>
<td>18 (56)</td>
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<td>8 (27)(^a)</td>
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<td>3 (20)</td>
<td>1 (7)(^h)</td>
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<td>1 (50)</td>
<td>2 (100)</td>
<td>not excluded</td>
</tr>
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<td>7 (78)</td>
<td>6 (67)</td>
<td>8 (89)</td>
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<td>8 (89)(^d)</td>
<td>7 (78)(^a)</td>
<td>4 (44)(^a)</td>
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<td>5 (33)(^d)</td>
<td>9 (60)(^d)</td>
<td>13 (87)(^d)</td>
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<tr>
<td>starter culture</td>
<td>47</td>
<td>10 (21)(^d)</td>
<td>23 (49)(^f)</td>
<td>23 (49)(^d)</td>
<td>15 (32)(^d)</td>
</tr>
<tr>
<td>blood</td>
<td>219(^k)</td>
<td>23 (91)(^c)</td>
<td>141 (64)(^f)</td>
<td>71 (32)(^d)</td>
<td>not excluded</td>
</tr>
<tr>
<td>various medical samples</td>
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<td>14 (24)</td>
<td>46 (79)</td>
<td>36 (62)</td>
<td>8 (14)</td>
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<td>feces/throat healthy volunteers</td>
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<td>3 (30)</td>
<td>4 (40)</td>
<td>9 (90)</td>
<td>1 (10)</td>
</tr>
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<td>environment</td>
<td>6</td>
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<td>5 (83)(^d)</td>
<td>2 (33)(^d)</td>
<td>0(^d)</td>
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<td>total medical isolates</td>
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<td>281/1008 (28)</td>
<td>397/649 (61)</td>
<td>194/422 (46)</td>
<td>9/73 (12)</td>
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<td>total commensal isolates</td>
<td>186</td>
<td>27/141 (19)*</td>
<td>48/104 (46)*</td>
<td>62/129 (48)</td>
<td>18/106 (15)</td>
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</table>

Note: data are no. (%).\(^a\) determined on agar plate with human blood, \(^b\) determined on agar plate with human blood and with a DNA probe, \(^c\) determined on agar plate with horse blood, \(^d\) determined with PCR or DNA probe, \(^e\) determined on agar plate with gelatin, \(^f\) determined on agar plate with gelatin and an agar plate with skim milk, \(^g\) determined with clumping assay, \(^h\) determined with clumping assay, western blot and DNA probes, \(^i\) 12 H\(^+\) and gentamicin R isolates were identical on PFGE, \(^j\) hemolysin determined on 211 isolates, \(^k\) determined with ribotyping, \(^*\) \(P \leq 0.05\) (\(x^2\) test).
through prolonged intracellular survival in macrophages and finally, Agg plays a role in adhesion to and internalization in epithelial cells a first step to infection.

**Enterococcal surface protein**
The Esp was initially identified in *E. faecalis* MMH594, a clinical isolate that caused multiple infections in a hospital ward outbreak. Esp is a large cell-surface-localized protein that has similar structure to other streptococcal surface proteins that have be shown to be virulence determinants and contribute to immune evasion through their different number of repeat motifs (217). Esp was also found to be part of a pathogenicity island that codes for most known auxiliary traits that enhance virulence and a number of additional previously unknown genes that are rare in non-infection-derived isolates (215). In an animal model of ascending urinary tract infection, significantly higher numbers of *E. faecalis* expressing Esp were recovered from the bladder and urine but not from the kidney compared to an Esp negative strain. However, no difference in histopathological changes was seen between the two strains, suggesting that Esp only contributes to maintenance and colonization of *E. faecalis* in acute urinary tract infections and thus suggesting an adhesive role for this protein (216). Furthermore, analysis of the relationship between the presence of Esp and biofilm formation showed a high association with the biofilm formation capacity on polystyrene surfaces (235). Table 1 shows an overall significant difference in the prevalence of Esp among medical and commensal *E. faecalis* isolates, confirming its role in pathogenesis.

**Adhesin to collagen of *E. faecalis***
Adhesion of bacteria to host cells or extracellular matrix proteins is the initial step in infection. Many different studies to the enterococcal adherence to extracellular matrix components, fibronectin, collagen(s) and laminin are performed. However, these studies showed conflicting results depending on the strains used, the experimental protocol or the growth conditions (136, 218, 220, 228, 266, 272). Xiao et al. (266) demonstrated an increase in binding to immobilized laminin and collagen types I and IV when *E. faecalis* was grown at 46 °C but not at 37 °C. Searching the genome database of *E. faecalis* revealed a protein that showed significant similarities with the collagen-binding protein, Cna, of *S. aureus*. This protein was given the name adhesin to collagen of *E. faecalis* (Ace) (203). Disruption mutation studies of the *ace* gene showed reduction in the 46 °C growth-elicited binding to collagen type I and IV and laminin (179, 180). However, the role of this protein in infection remains unclear as the *ace* gene was found in 62 of 62 *E. faecalis* isolates regardless of their origin (62). Results from Nallapareddy et al. (180) indicate that Ace might be produced during infection because antibodies to Ace were found in 90 % of sera from endocarditis patients.

**E. faecalis antigen A**
The *E. faecalis* antigen A (EfaA) was identified by two groups through screening of different surface proteins with sera from endocarditis patients (143, 267). In a mouse peritonitis model, mice infected with an *E. faecalis* mutant that lacked the EfaA showed more prolonged survival compared to mice infected with the parent strain (222). However, the contribution of EfaA to pathogenesis could not be confirmed through a higher incidence in
infection derived isolates as the gene was found in 42 of 42 \textit{E. faecalis} isolates from both infection and healthy volunteers (222) and also in Table 1 EfaA was found in up to 90 \% of medical and commensal \textit{E. faecalis} isolates although total numbers are low.

\textit{Extracellular polysaccharides}
Different research groups found several polysaccharides that might play a role in infections. Cloning a genomic library of \textit{E. faecalis} in cosmid vectors and screening with rabbit immune serum and serum from patients with enterococcal endocarditis revealed a non protein antigen that was identified as a carbohydrate (267, 268). Further analysis of this cosmid clone identified a gene cluster, enterococcal polysaccharide antigen (\textit{epa}), encoding homologues of many genes involved in polysaccharide biosynthesis (267-269). Evidence showed that this \textit{epa} gene is widespread among \textit{E. faecalis} isolates and increases resistance to neutrophilic killing of \textit{E. faecalis} (232). Mutants with disrupted genes showed delayed killing and a higher lethal dose in a mouse peritonitis model. Furthermore, the antigen was detected in two mucoid \textit{E. faecalis} strains from chronic urinary tract infections (269). Another study by Huebner et al. (111) isolated and chemically characterized a capsular polysaccharide antigen by immunization of rabbits with killed \textit{E. faecalis} strains. This capsular polysaccharide antigen was found on approximately one third of 15 \textit{E. faecalis} strains. Furthermore, they showed that antibodies against the capsular polysaccharide antigen were protective in a mouse model of infection (239). Another capsular polysaccharide was detected by Hancock et al. (104). Evidence for the role of this capsular polysaccharide in pathogenesis was given by the fact that insertional inactivated mutants were enhanced susceptible to phagocytic killing and were significantly compromised in their ability to persist in lymphatic tissue in vivo (104).

\textit{Cytolysin}
The cytolysin/hemolysin of \textit{E. faecalis} is a secreted, posttranslational modified protein that is regulated by a two-component regulatory system via a quorum sensing mechanism (103). The cytolysin is distantly related to streptolysin S and also to members of a class of bacteriocins known as the lantibiotics, it lyses human, rabbit and horse erythrocytes and is active against many Gram-positive bacteria (10). Details of the discovery and characterization of the working mechanism are described in a recent review (43). The encoding operon for cytolysin is either encoded on the sex-pheromone plasmids or integrated into the chromosome within a pathogenicity island (115, 215). It has been hypothesized that the cytolysin may influence the pathogenesis of infection through lysis of erythrocytes or the destruction of other host cells (43). Furthermore, the cytolysin may impact pathogenesis by lysing PMNs and macrophages as was shown by Miyazaki et al. (158). Isogenic mutants of the cytolysin operon were used to study the role of the cytolysin in the pathogenesis of infection in different animal models. Generally, these models showed an increased virulence of cytolytic \textit{E. faecalis} compared to non-cytolytic \textit{E. faecalis}. First, Ike et al. (117) examined the contribution of cytolysin to pathogenicity in intraperitoneally infected mice. Strains exhibiting the normal cytolysin phenotype were significantly more virulent than the non-cytolytic insertion mutants. A mutant plasmid with an increased cytolytic phenotype rendered host strains more virulent than the wild-type cytolytic
enterococci in mice. The role of cytolysin in the pathogenesis of peritonitis in mice was confirmed in different studies (67, 223). Dupont et al. (67) found the lowest 50 % lethal dose (LD50) with strains of *E. faecalis* expressing the cytolysin or a combination of the cytolysin with Agg. In addition, Singh et al. (223) reported a 35-fold lower LD50 with *E. faecalis* producing cytolysin in a mouse peritonitis model with added sterile rat fecal extracts. Cytolysin has also been shown to contribute to the mortality due to endocarditis in a rabbit endocarditis model (39). Mortality was significantly increased in animals given *E. faecalis* expressing Agg and cytolysin (55 %) compared with *E. faecalis* expressing only Agg (15 %) or only cytolysin (no mortality) (39). In an endophthalmitis model, *E. fecalis* positive for cytolysin destroyed the neural tissue of the retina and its architecture whereas non-cytolytic strains produced few or no destructive changes (124). Furthermore, in a *C. elegans* model, the cytolysin was observed to enhance nematode killing by *E. faecalis* (91). An indication of the virulence of cytolysin in human infections was the observation that patients with bacteremia with a cytolytic strain had a fivefold increased risk of mortality. In this study, the cytolytic strains were also gentamicin resistant (114). However, Caballero-Granado et al. (35) reported that the mortality associated with high-level gentamicin resistant *E. faecalis* bacteremia was not significantly different from mortality associated with gentamicin susceptible strains. The role of cytolysin in infection is confirmed by the epidemiological data in Table 1 which show a significant difference in the incidence of cytolytic *E. faecalis* among commensal and medical isolates.

Proteases

Two secreted proteases have been described for *E. faecalis*, namely gelatinase and serine protease, and both are regulated by the *fsr* system that is described below. The gelatinase was shown to hydrolyze gelatin, collagen, casein, lactoglobulin, porcine myofibrillar proteins, porcine sarcoplasmic proteins, and other small biologically active peptides (149). The gelatinase might therefore contribute to virulence by degradation of specific host proteins and damaging host tissue. Experiments in animals confirmed the role of gelatinase in virulence. In a rabbit endocarditis model, proteolytic *E. faecalis* strains were associated with partial dissolution of the vegetation and a more severe clinical picture (100). In two mouse peritonitis models the gelatinase positive strains showed a lower LD50 than the gelatinase negative strain (67, 223), however this effect was not observed in a rat peritonitis model (67). Finally, an *E. faecalis* deletion mutant defective in gelatinase production was attenuated in its ability to kill *C. elegans* (221). Table 1 shows a significant difference between the incidences of gelatinase positive isolates from medical and commensal sources thus indicating a possible role in infection in humans.

The serine protease was detected by sequencing the regions downstream of *gelE*, the gene coding for the gelatinase (199). Northern blot analysis revealed that *gelE* and the gene for the serine protease (*sprE*) were cotranscribed (199). However, experiments in a mouse peritonitis model with a *sprE* mutant showed a prolonged survival of the mice compared to survival after infection with the parent strain, indicating that the serine protease is independently important for infection in this model (199).

Sequencing the regions upstream of the *gelE* gene revealed three open reading frames that were named the *fsr* (*E. faecalis* regulator) locus. The Fsr system is a quorum-sensing system
that regulates the expression of gelatinase and serine protease (200). Disruption mutants of all three open reading frames of the fsr locus showed a prolonged survival of the mice compared to the parent strains in a mouse peritonitis model (199, 221). In addition, mutants in one of the open reading frames of the fsr locus were attenuated in their pathogenicity in the rabbit endophtalmitis model (178) and C. elegans model (91, 221). The role of the Fsr system in virulence was confirmed by a retrospective epidemiological study that found fsr in a significant higher percentage of endocarditis isolates versus of stool isolates, however this difference was not found for other clinical isolates (195).

**Nosocomial infections with E. faecalis**

*E. faecalis* is a frequent cause of hospital acquired infections. In a recent study on the frequency of isolation of different bacterial species from patients in intensive care units in Europe, *E. faecalis* was found in about 4 % of the isolates ranking number 8 in the list of most frequently isolated microorganisms (79). In many of the nosocomial enterococcal infections local and/or systemic immunity is compromised for example by catheterization, surgery in areas that are normally colonized with enterococci or medical immune suppression after transplantation (8, 40). A combination of these factors is present in many of the critical ill patients at intensive care units, for example in liver transplant patients that are often considered especially vulnerable to enterococcal infections (248, 250).

**Nosocomial transmission and acquisition of enterococci**

The intrinsic robustness of *E. faecalis* may allow members of this species to survive for extended periods of time, leading to its persistence and nosocomial spread. *E. faecalis* can grow at 10 to 45 ºC, in 6.5 % NaCl, in the presence of 40 % bile salts and over a broad range of pH (74). Originally, enterococcal infections were thought to arise from a patient’s own endogenous flora or to be introduced into the abdomen during transplant surgery or its complications (6, 73). However, molecular epidemiological studies provided evidence for epidemic spread of enterococci in a hospital setting and nosocomial acquisition of enterococci (7, 131, 171). Zervos et al. (274, 275) were among the first to demonstrate nosocomial acquisition and nosocomial spread of gentamicin-resistant *E. faecalis*, using plasmid content as an epidemiologic marker. They suggested that the transmission might have been through the hands of personnel (275). Later, Livornese et al. (141) were the first to document an inanimate object, in this case rectal thermometer probes, as the mode of transmission of a vancomycin resistant *E. faecium*. Removal of the rectal thermometer probes resulted in termination of the outbreak. These reports were followed by many reports on nosocomial outbreaks and transmission of antibiotic resistant enterococci (131, 172, 191). Risk factors for the nosocomial acquisition of enterococci that are described in the majority of studies are: previous antimicrobial therapy, duration of hospitalization, severe underlying disease, or invasive procedures (20, 131). Nosocomial enterococcal acquisition and infection are often due to superinfection after the use of antibiotics with little or no anti-enterococcal activity like cephalosporins or quinolones (38, 273). Prevention
and control of transmission include the controlled use of antibiotics, active surveillance cultures to identify the reservoir for spread and stringent application of recommended contact precautions (171).

A variant of the esp gene was detected in all epidemic vancomycin-resistant *E. faecium* in hospitals but not in non-epidemic animal isolates (260). This indicates that the surface protein Esp is associated with enterococcal colonization and spread. Analysis of the mechanism underlying the influence of this surface protein on enterococcal transmission might lead to new ways to prevent colonization and transmission.

**Clinical manifestation of *E. faecalis* infections**

**Urinary tract infections**
The most frequent hospital infections caused by enterococci are urinary tract infections. Enterococci are found in up to 15 % of urine isolates, ranking only second after *Escherichia coli* (23, 24, 78, 142). Risk factors for enterococcal urinary tract infection have been identified as urinary tract instrumentation or catheterization, other genitourinary tract pathology and previous use of antibiotics, especially cephalosporin (165). The influence of instrumentation on infection and colonization was illustrated by a study by Gross et al. (98). In this study, 30 of 34 urinary tract infections associated with enterococci were preceded by urinary tract instrumentation and not related to antecedent antibiotic therapy. The clinical manifestations of enterococcal urinary tract infection are similar to those of other organisms. A reliable diagnosis of urinary tract infection can be difficult because enterococci are opportunistic pathogens that can also be colonizers or cause asymptomatic bacteriuria. Different studies were performed to investigate the role of surface proteins of *E. faecalis* in the interaction with uroepithelial tissue (132, 216). Kreft et al. (132) showed a potential role for Agg in the adhesion of enterococci to renal epithelial cells. In addition, Esp was shown to contribute to colonization and persistence of *E. faecalis* at the urinary tract (216).

**Endocarditis**

*E. faecalis* is associated with endocarditis in a significant percentage of cases. In a 2-year study of all endocarditis cases in The Netherlands *E. faecalis* accounts for about 8 % of the isolates, thereby being the third most common species following *Staphylococcus aureus* and *Streptococcus sanguis* (241). The probable source of infection in cases of enterococcal endocarditis is the genitourinary tract (14 to 70 % of cases), followed by the gastrointestinal tract (3 to 27 % of cases) and dental tract (2 to 12 % of cases) (156). Manipulation, surgery or disease in these areas is generally the immediate cause of the infection. Enterococci can infect normal valves as well as those with pre-existing damage or pathology. Usually, native valve enterococcal endocarditis is a subacute infection with a more protracted course (138, 148). In patients with valve replacements, *E. faecalis* is one of the predominant species in late prosthetic valve endocarditis (241). Mortality due to enterococcal endocarditis has been quoted as being between 10 and 40 % (99, 151, 156).

Different enterococcal virulence mechanism might be involved in the pathogenesis of endocarditis (153). As noted above, enterococci cultured in serum exhibit enhanced binding to Girardi heart cells (101, 102). Furthermore, experiments with *E. faecalis* endocarditis in
animal models suggest that both cytolysin and Agg are associated with increased mortality and/or vegetation weight (39, 213) and proteases are associated with a more severe clinical picture (100).

**Bacteremia**
About 5 to 10 % of bloodstream isolates is *E. faecalis*, thereby *E. faecalis* ranges number 6 in the list of causative microorganisms of bacteremia (77, 142). However, up to 45 % of enterococcal bacteremias are polymicrobial (90, 148, 219). Enterococcal bacteremia is much more common than enterococcal endocarditis. The proportion of significant episodes of enterococcal bacteremia that was deemed to represent endocarditis ranged from 1 to 30 %, where the percentage generally depended on the definition of bacteremia in the underlying study (156, 169). The sources of bacteremia without endocarditis are similar to the sources of endocarditis as mentioned above with soft tissue infections and burn wound infections added to the list. However, for a significant number of bacteremias no source could be identified (90) which stimulates the speculation that the translocation of enterococci across an intact intestinal epithelial barrier might lead to many of these bacteremias (255).

The reported mortality among patients with enterococcal bacteremia has been high (up to 45 %), most probably because of severe underlying complicating factors (138, 148, 150, 219). Only few comparative studies on the attributable mortality and morbidity associated with enterococcal bacteremia are published and their results are contradictory partly due to differences in design of the study. In a prospective, matched case-control study, Caballero-Granado et al. (34) found no increased mortality but an increased duration of stay among patients with enterococcal bacteremia. On the other hand, Landry et al. (137) reported an attributable mortality rate due to enterococcal bacteremia of 31 % in a retrospective cohort study. Factors predicting a fatal outcome of enterococcal bacteremia are: (i) infection with a resistant organism, (ii) inadequate antimicrobial therapy, (iii) prior use of antimicrobial therapy, (iv) severe underlying disease, (v) use of intravascular catheter, (vi) renal dialysis, and (vii) prior need for mechanical ventilation (34, 137, 247). Risk factors for the development of enterococcal bacteremia are: (i) neutropenia, (ii) urinary catheter, and (iii) previous administration of cephalosporins and imipenem (33). These studies underline the importance of appropriate use of antibiotics with no or little anti-enterococcal activity.

One epidemiological study by Huycke et al. (114) showed a relation between the presence of the virulence trait cytolysin and mortality due to enterococcal bacteremia. However, this relation could not be confirmed in a recent study investigating the association between the presence of enterococcal virulence factors and mortality among patients with enterococcal bacteremia (247).

**Intra-abdominal infections**
Although enterococci can be isolated in a significant number of intra-abdominal infections, usually as part of a polymicrobial infection, their role in these infections is controversial (55). Animal models of bacterial peritonitis showed that enterococci alone did not cause any abscess formation (186), but a mixed inoculation of *E. faecalis* and other aerobe or anaerobe bacteria resulted in death and abscess formation suggesting a synergistic effect of *E. faecalis* in the pathogenesis of bacterial peritonitis (152, 162, 163, 186). This finding is
underscored by the fact that antibiotics that lack activity against enterococcus can often be employed successfully in intra-abdominal infections, even when enterococci are present as part of the polymicrobial flora (227, 262). However, others suggested that the role of E. faecalis in experimental peritonitis might depend on the presence of virulence factors (67, 117). Furthermore, several reviews on bacteremia have revealed that the source of enterococcal bacteremia is intra-abdominal infection in a number of cases (90, 148, 150, 219) and some clinical report describe enterococci as the sole cause of intra-abdominal infections (48, 273). Finally, Burnett et al. (29) reported enterococcus as a predictor of treatment failure in complicated intra-abdominal infections. Predisposing factors for the presence of enterococcus in intra-abdominal infections include age, severity of underlying disease, hospital length of stay, and the presence of foreign bodies e.g. drains and prior antibiotic use (29, 40, 273). A problem in evaluating the importance of E. faecalis in the pathogenesis of intra-abdominal infection in humans is that surgical drainage is usually part of the therapy, and drainage alone may be enough to achieve cure.

Skin and soft tissue infections

E. faecalis accounts for up to 5 % of isolates from skin and soft tissue infections (78, 126). Enterococci generally cause infections only in previously damaged tissues and are not apparently responsible for primary cellulites. Especially in wound infections after abdominal surgery enterococci are frequently cultured (8). However, since enterococci from skin and soft tissue infections are frequently cultured in association with other pathogens, their role in pathogenicity is unclear.

Biomaterial associated infections

Many nosocomial enterococcal infections are associated with medical devices such as intravascular or urinary catheters, bile drains and prosthetic heart valves (54, 75, 97, 270). Biomaterial-centered infections are initiated by bacterial adhesion and biofilm formation on the indwelling device. This biofilm can be a source of persistent infection, because the biofilm mode of growth protects the adhering bacteria against the host defense and the action of antimicrobial agents (45). These biomaterial related infections cause significant morbidity and can be very difficult to treat, often removal of the device is necessary (210). Recently, Sandoe et al. (211) reported that E. faecalis isolated from intravascular catheter related bloodstream infections produced significant more biofilm compared to bloodstream isolates from other sources. This suggests that the ability to form a biofilm might be a marker of virulence. By use of a polystyrene microtiter plate biofilm assay, Toledo-Arana et al. (235) confirmed the association of Esp and biofilm formation. However, also E. faecalis without Esp or Agg was shown to be able to produce biofilms (133). In this study, the production of gelatinase enhanced the biofilm formation of E. faecalis (133) but this was not confirmed in another study (127). Furthermore, different environmental conditions can influence the ability of E. faecalis to adhere and produce biofilms. Adherence of E. faecalis to different biomaterials increased by growth in the presence of 10 % serum or sub-inhibitory concentrations of ampicillin and vancomycin (85, 86). Biofilm formation also increased in the presence of a nutrient poor medium but decreased with higher osmolarity of the medium (133). Possibly, the different virulence factors of E. faecalis work in concert
in a biofilm; the surface proteins Esp and Agg function to mediate clumping or adherence leading to biofilm formation and thereby increasing the local density of bacteria to the level necessary to induce the cell density dependent expression of cytolysin or gelatinase regulated by the Fsr system (95).

Concluding remarks and future directions

*E. faecalis* is an emerging pathogen with an increasing incidence among hospitalized patients. Therefore *E. faecalis* attracts more and more attention from medical microbiologists and infectious disease specialists. Many possible virulence traits of *E. faecalis* are studied by use of animal models, in vitro models and isolates from different groups of patients. However, animal and in vitro models of infection are hampered by the fact that they often do not represent the human situation and different studies frequently show conflicting results. Furthermore, studies to the incidence of virulence factors among different patient groups are blurred due to the fact that patients with high immune suppression are also susceptible to commensal strains without virulence factors. Despite these numerous investigations, the mechanism of virulence of *E. faecalis* is still not completely understood. Results indicate a role for the known virulence traits of *E. faecalis*. However, other unknown factors might also play a role because enterococci without any of the known virulence factors can cause serious infections and some aspects of pathogenesis can not be explained with the present theories.

The recent publication of the entire genome of *E. faecalis* V583 (192) will lead to the detection of new virulence traits. Analysis of these new and old virulence traits with the use of newly derived methods like DNA micro arrays might lead to new insights in the pathogenesis of infection and the discovery of ways to prevent or treat these infections without the extensive use of antibiotics.

Scope of the thesis

At the University Hospital Groningen, about 55 liver transplants are performed every year. Liver transplant recipients are very vulnerable to infections and bacteria are the most common cause, with a reported incidence ranging from 30 to 65 % (9, 93, 135, 146, 249, 264). Most bacterial infections occur in the first 4 weeks after the transplantation (9, 146, 193, 248). Gram positive cocci are the major organism isolated from liver transplant patients with enterococci accounting for about 10 % of the bacterial episodes (189, 248, 249, 264). Risk factors contributing to bacterial infection after liver transplantation are prolonged surgery, bleeding, use of choledochojejunostomy (roux-en-Y) instead of a choledochocholedochostomy (duct-to-duct) for biliary anastomosis, repeated abdominal surgery and CMV infection (189, 194). Furthermore, the isolation of predominantly Gram positive microorganisms might be associated with the prophylactic use of antibiotics with no anti-enterococcal activity (146, 189, 193, 276).

The concern about *E. faecalis* as an emerging pathogen, its role in infections among liver
transplant patients and the substantial morbidity and mortality associated with these infections led us to study the pathogenesis of *E. faecalis* infections in this group of patients. The general aim of this thesis was to gain insight in the pathogenesis of *E. faecalis* infections with special interest in liver transplant patients. The starting point for our investigation was the microorganism and to find virulence factors and mechanisms of *E. faecalis* that might lead to infections or spread. To achieve the general aim of this thesis, the outline of our studies pertaining to the underlying questions will be addressed in the subsequent chapters. **Part I** focuses at the epidemiology and the role of virulence factors of *E. faecalis* in infections in liver transplant patients. In **Chapter 2**, the differences in prevalence of virulence factors and genomes (genogrouping) among isolates from liver transplant patients and isolates from other patient groups or healthy volunteers are analyzed. The central question in this chapter was whether *E. faecalis* from liver transplant patients is different from isolates from other sources. In **Chapter 3**, the epidemiology of *E. faecalis* among liver transplant patients is investigated. In **Chapter 4**, the development and evaluation of a rapid non-cultural method to detect enterococcal infections and to study their spatial distribution in the sample is described.

In **Part II** of the thesis the mechanisms of adhesion of *E. faecalis* to different biomaterials are studied. Biofilms formed on biomaterials inserted into the human body are a frequent source of infection. Adhesion is the first step in biofilm formation. In **Chapter 5**, the role of the surface proteins Agg and Esp of *E. faecalis* on its adhesion to different biomaterials investigated and a proposal for the adhesion mechanism of *E. faecalis* is given. In **Chapter 6**, the molecular interaction forces between *E. faecalis* expressing Agg is measured with atomic force microscopy and the influence of antibodies to Agg on these forces is investigated. In **Chapter 7**, the influence of the growth in the presence of (ox) bile of *E. faecalis* on the numbers of bacteria adhering to different biomaterials and the cell surface properties involved in initial adhesion is studied. In **Chapter 8**, the results and conclusions of this thesis are summarized and discussed.