Pathogenesis of nosocomial infections with Enterococcus faecalis

Waar, Karola

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Summarizing Discussion
In the normal healthy host the bacterium *Enterococcus faecalis* colonizes the gastrointestinal tract and seldom causes infections. However, in hospitalized patients this microorganism is at the increase as a cause of infections and liver transplant patients are especially vulnerable to these infections. Why these patients are more frequently infected with *E. faecalis* compared to other patients is not clear. In the pathogenesis of infection generally two factors play a role. On the one hand, the immunity that normally protects the patients can be diminished by immune suppressive treatment and medical interventions e.g. surgery or implantation of an indwelling biomaterial that will break the natural defense of the skin and mucous membranes. On the other hand, bacteria that are normally not pathogenic in the healthy host can acquire special traits that enhance virulence or increase adhesion to biomaterials and therefore cause infections in the hospital environment. The aim of this thesis was to gain insight in the pathogenesis of infections with the bacterium *E. faecalis* with special interest in liver transplant patients. The focus of our investigation was the microorganism and to find traits and mechanisms of *E. faecalis* that might lead to more severe infection, spread or biomaterial associated infections. In Part I of this thesis the investigations to the role and epidemiology of *E. faecalis* in infections in the host are described, Part II focuses on biomaterial centered infections with *E. faecalis* and the role of some surface proteins of *E. faecalis*. In the current chapter, the different investigations that were conducted to answer these questions are summarized, the results are discussed, the shortcomings are described and future directions are given.

Many different traits of *E. faecalis* that possibly play a role in infections are described in literature (Chapter 1). To study the possible role of these virulence factors in infections in liver transplant patients, we compared the prevalence of these traits among *E. faecalis* isolates from liver transplant patients to the prevalence among isolates from other patients and healthy volunteers (Chapter 2). The prevalence of the virulence factors cytolysin (hemolysin), gelatinase, enterococcal surface protein (Esp) and the genes asa1 and asa373 encoding two forms of aggregation substance (Agg) was determined among isolates from liver transplant patients (133 isolates), blood culture isolates from non-liver transplant patients (66 isolates) and a control group of non-clinical enterococci from feces of healthy volunteers (47 isolates). To exclude multiple identical isolates, amplified fragment length analysis (AFLP) was performed. In addition, the AFLP analysis furthermore discerned 11 genogroups in which the strains shared > 80 % of the restriction fragments. The majority of the isolates clustered in three of these genogroups that respectively consisted of blood cultures, isolates from liver transplant patients or isolates from feces of healthy volunteers. Combination of the genogrouping and the presence of virulence factors showed that cytolysin (hemolysin) and asa1 might be associated with infections, because they were mainly found among isolates from the genogroups representing blood cultures and liver transplant patients. Furthermore, Esp might be associated with colonization because the gene encoding the protein is mainly found in isolates from the genogroups represented by fecal isolates from healthy volunteers or liver transplant patients.

Studying the role of virulence factors based on their incidence among isolates from different groups of patients can be difficult because almost certainly the collection of isolates will be a mixture of virulent and non-virulent isolates, especially among liver transplant patients that are immune compromised and might be vulnerable to both virulent and non-virulent
strains. Future studies possibly should also involve other groups of patients to rule out the possibility of inclusion of non-virulent strains. Very interesting in this study is the finding of different genogroups among the *E. faecalis* isolates related to the different hosts that were studied. This indicates that some enterococci might have special traits that allow them to survive in a special environment, e.g. the hospital. The enterococci isolated from this environment are therefore genetically more related compared to enterococci from other environments. This finding opens the opportunity to a new approach in the study of the pathogenesis of *E. faecalis* infections. Comparing the genomes of the different genogroups might lead to the identification of certain traits that the enterococci need to survive in a certain environment. Interference with these traits or colonization of these environments with less virulent traits might lead to ways to prevent infections with *E. faecalis*.

The AFLP analysis of the *E. faecalis* isolates from liver transplant patients described in Chapter 2 revealed one particular epidemic clone that colonized 23 of the 43 liver transplant patients over a 15 months period. Chapter 3 describes the epidemiology and characteristics of this and other epidemic isolates on the liver transplant ward. Among the 133 *E. faecalis* isolates from liver transplant patients that are described in Chapter 2, 15 different AFLP types could be identified with 90 % similarity which was considered the cut-off point for identical isolates. Nine of these groups contained isolates from more than one patient which might indicate transmission. In five of these groups transmission could be explained by the fact that patients carrying identical strains were staying in the same ward at the same time. Analysis of the characteristics of the epidemic isolates in this study showed that these isolates did not cause more infections compared to the non-epidemic isolates. Furthermore, the epidemic isolates were more resistant to the antibiotics gentamicin, erythromycin and ciprofloxacin but none of the isolates was resistant to the clinically important antibiotics vancomycin or amoxicillin. Reports about the transmission of enterococci susceptible to most antibiotics are scarce; therefore, the most important message of Chapter 3 is the description of the transmission of a susceptible *E. faecalis* strain. Knowledge about the transmission of these strains is important because enterococci are able to acquire resistant determinants relatively easily and unrecognized transmission of susceptible isolates may finally result in outbreaks of multi-resistant strains. Another important finding was that the epidemic clone was positive for the surface protein Esp. This confirms the role of Esp in transmission and spread and emphasizes the possibility of interference with transmission through interaction with Esp.

In Chapter 4 the design and testing of two DNA oligonucleotides specific for the 16S rRNA genes of respectively *E. faecalis* and *Enterococcus faecium* are described. When these oligonucleotides are labeled with a fluorescent dye they can be used for fluorescent in situ hybridization (FISH). FISH is a method to directly visualize and identify bacteria in a clinical sample without any culturing. Testing of the oligonucleotides on other enterococci showed that the *E. faecalis* probe only reacted with *E. faecalis* species. The *E. faecium* probe also hybridized with *Enterococcus hirae*, *Enterococcus mundtii* and *Enterococcus saccharolyticus* as could be expected from the sequence information. We further describe FISH protocols for the detection of *E. faecalis* and *E. faecium* in fecal samples, blood cultures and biofilms on indwelling devices. The studies that use the FISH for detection of enterococci are generally hampered by the fact that they are only focused on one material. By describing
the use of FISH on different clinical samples we show that the oligonucleotides can be broadly applied to different materials. The most interesting fact of this report is that we were able to show that the enterococci indeed are present on a biofilm attached to a bile drain. This opens the possibility to study the distribution of enterococci in biofilms that are formed in vivo.

In Part II of this thesis the focus shifts towards biomaterial related infections with *E. faecalis*. When a biomaterial is introduced into the body it frequently gets colonized with bacteria. These bacteria form a biofilm on the biomaterial. This biofilm can be a source of persistent infections because bacteria in a biofilm are generally more resistant to antibiotics. The first step in biofilm formation is adhesion of the bacteria to the material. Insight in the mechanisms of adhesion can lead to the development of methods to prevent this adhesion and therefore biofilm formation. Our investigations were focused on liver transplant patients and in these patients the bile drain that is used to divert the bile outside the body is a frequent source of infection. Therefore we chose to study the adhesion of *E. faecalis* to different bile drain materials that are used at the University Hospital Groningen.

First, the role of three surface proteins of *E. faecalis* (Esp and two forms of Agg; Asa1 and Asa373) in the adhesion to silicone rubber, fluoro-ethylene-propylene and polyethylene was examined (Chapter 5). Four isogenic *E. faecalis* strains with and without Agg and one strain expressing Esp were used. With the use of a parallel plate flow chamber the kinetics of initial adhesion of the strains to the materials were studied in detail. The results indicate that the surface proteins of *E. faecalis* play a key role in the adhesion and increased the total number of adhering bacteria. However, the different surface proteins increased the adhesion of *E. faecalis* through different mechanisms. Analysis of the distribution of the enterococci after the initial adhesion showed that the enterococci expressing Agg adhered in higher numbers through mechanisms of positive cooperativity, which means that adhesion of bacteria enhances the probability of adhesion of other bacteria near these bacteria. Enterococci positive for Esp also adhered in high numbers, but did not utilize positive cooperative mechanisms in their adhesion to the biomaterials. Possibly, Esp increased the strength of the direct interaction between *E. faecalis* and the material. These findings indicate some possibilities to design drains that prevent biomaterial related infections. This might be through interference with the positive cooperativity between adhering enterococci, as resulting from the expression of Agg e.g. by adding antibody to Agg or through interference with the interaction between surface proteins and materials e.g. by changing the surface properties of the biomaterial.

In the search for ways to prevent biomaterial related infections, we investigated whether antibodies to Agg can interfere with the positive cooperativity between *E. faecalis* expressing Agg (Chapter 6). The interaction forces between *E. faecalis* strains with and without Agg (Asa1 and Asa373) were measured at a molecular level by use of atomic force microscopy (AFM). *E. faecalis* expressing Agg showed nearly two-fold higher interaction forces between bacterial cells than a strain lacking Agg. Interestingly, these strong interaction forces between the strains with Agg could be reduced by adsorption with antibodies against Agg. Comparison of the results of the AFM interaction forces with the positive cooperativity after adhesion to a biomaterial in a parallel plate flow chamber showed that in the absence of strong interaction forces between the bacteria, positive
cooperativity was absent too. The absence of positive cooperativity further resulted in a lower total number of adhering bacteria. The results of this study show that specific antibodies influence the interaction forces between \textit{E. faecalis} and that these interaction forces have a direct impact on the way these bacteria colonize a biomaterial surface. Furthermore, a role in the prevention of biomaterial related enterococcal infections might be granted to these antibodies.

Evidence from literature indicates that \textit{E. faecalis} is resistant to the bactericidal effects of bile and this resistance is induced by expression of a large number of stress proteins (76). Furthermore, some reports indicate that bile plays a role in the adhesion of bacteria (230, 256). Therefore, we investigated the influence of growth in the presence of bile on the adhesion of \textit{E. faecalis} to bile drain materials and measured the physicochemical properties of the bacterial cell surface (Chapter 7). Because the previous study showed that the surface proteins Esp and Agg played a role in adhesion, we used strains expressing these proteins. After growth in the presence of bile, the strains were generally more hydrophobic and zeta potentials were more negative than when the strains were grown in the absence of bile. Moreover, \textit{E. faecalis} not expressing Agg or Esp and \textit{E. faecalis} with Esp on its surface adhered in an up to twofold-higher number after growth in bile. \textit{E. faecalis} expressing Agg did not adhere in higher numbers after growth in bile, possible because they mainly adhere through positive cooperativity and less through direct interactions with a substratum surface. The results of this study show that growth in bile can increase the virulence of \textit{E. faecalis} as adhesion of bacteria is the first step to biomaterial related infection. Analysis of the effect of bile on the expression of surface proteins might lead to new ways to prevent bile drain related infections.

The parallel plate flow chamber model that was used in the studies to biomaterial related infections is a very adequate model to study the adhesion mechanisms in detail. The disadvantage of this in vitro model is that it is very difficult to imitate the in vivo situation. In vivo many more factors influence the adhesion and biofilm formation than the variables included in our study. In the study to the influence of bile on the adhesion, not only growth in bile but also the presence of bile during the adhesion process and the coating of bile drain with bile components prior to the adhesion might influence the adhesion. These variables could be the subject of future studies. Furthermore to the study of the use of antibodies to prevent adhesion to biomaterials it should be noticed that coating the bacteria with antibodies before adhesion is not a measure that can be performed in vivo. However, the study of isolated variables that influence adhesion is the only way to gain more insight in the exact mechanisms of adhesion. The results of these experiments will have to be confirmed in an in vivo model.

**General conclusions**

- \textit{E. faecalis} isolates from different groups of patients and healthy volunteers can be divided into different genogroups with $> 80 \%$ shared restriction fragments and each genogroup with specific predominant virulence factors.
- Virulence factors cytolysin (hemolysin) and Asa1 might be associated with infection and Esp with colonization and spread between (liver transplant) patients.
- *E. faecalis* with different surface proteins adheres to the substratum through different mechanisms. Enterococci expressing Agg adhere in high numbers through positive cooperativity between adhering bacteria but enterococci positive for Esp do not adhere through this mechanism.
- Antibodies to Agg interfere with the interaction forces between *E. faecalis* expressing Agg as measured with AFM and these forces have a direct impact on the way these bacteria colonize a biomaterial surface.
- Growth in the presence of (ox) bile changes the surface properties of *E. faecalis* and increases adhesion of some particular strains.

**Concluding remarks and future directions**

In this thesis, we analyzed the role of the different virulence factors of *E. faecalis* known in literature in the pathogenesis of infections. We investigated the prevalence of virulence factors in *E. faecalis* isolates from different hosts as well as their role in biomaterial related infections. The results showed that the virulence factors cytolysin (hemolysin) and the Asa1 form of the surface protein Agg are involved in infection and the surface protein Esp is associated with colonization. In addition, the surface proteins Agg and Esp play a role in the adhesion to biomaterials. However, another important conclusion from this thesis is that also other factors are involved in the pathogenesis of infection; *E. faecalis* without any of the virulence factors which are known thus far also caused infections and strains without any of the studied surface proteins adhered to biomaterials.

Beside the bacterium also the host plays a role in the pathogenesis of bacterial infections. In modern medicine severely immune compromised patients are no exception, this makes the analysis of pathogenesis of opportunistic pathogens more complicated as it seems difficult to apply the postulates of Koch on these patients. When the immune defense of the host is compromised also non-virulent bacteria can cause infections and collections of isolates that cause infections in these patients might be a mixture of virulent and non-virulent isolates. Furthermore, evidence suggests that after admission to a hospital the gastrointestinal tract of patients quickly gets colonized with enterococci of nosocomial origin (234, 275). A factor that predisposes to nosocomial colonization with enterococci is the use of antibiotics with little or no anti-enterococcal activity. The question raised is why the indigenous enterococci do not take advantage of their presence and immediately fill the niches that are left by the bacteria killed by the antibiotic regime. The nosocomial enterococci that initially are outnumbered by the indigenous enterococci finally colonize the gastrointestinal tract and cause infections, this suggests that these nosocomial enterococci poses traits that give them advantages in this environment (96). The trait that nosocomial enterococci poses is not necessarily antibiotic resistance because in this thesis we showed that also enterococci susceptible to the clinically relevant antibiotics can spread between hospitalized patients. This implicates that in future the pathogenesis of a “commensal pathogen” like *E. faecalis* may not only be studied by direct search for straight virulence factors but also by analysis of factors that enable the bacterium to survive in an environment from where it can easily infect the host as soon as the immunity decreases. The environment that nosocomial
enterococci colonize before causing an infection might not necessarily be the gastro-intestinal tract of the patient but also the colonization of other niches e.g. the skin or respiratory tract of patients and the hands of personnel has to be taken into account. Colonization of this kind could be defined as ‘nosocomial colonization’, a term that would include the colonization of all sites from where the patient can be infected and that would also include competition with the indigenous flora that can be present on this site. The need for a redefinition of virulence that is implicated by the former statement was recognized by several infectious disease specialists (36, 251). They suggested a classification of microbial pathogenicity that takes into account the host immune response, allocating one class for microorganisms that only cause damage in situations of weak immune response (36). In addition, a classification for virulence genes was proposed that divides the genes into true virulence genes, virulence associated genes and genes essential for a pathogenic life-style (251). Genes that increase the ability of a microorganism to colonize the gastro-intestinal tract as described above could be classified as pathogenic life-style genes. However, in analogue to the different steps in pathogenesis, virulence factors could also be classified into classes that represent the different stages of pathogenesis. This would solve the problem of definition of virulence among opportunistic pathogens as these can have factors that are involved in the first steps of pathogenesis. Table 1 gives a suggestion of the classification of virulence factors and a suggested arrangement of the known virulence factors of *E. faecalis* over the different classes as far as the function of the virulence factor is known.

The adjustment of the definition of virulence as described above implies also a change in the infection models that are used to prove the role of virulence factors in pathogenesis. Up to now, many infection models include the determination of the lethal dose in animals or at least the development of infection in healthy animals. New infection models should be more directed towards earlier steps in pathogenesis e.g. colonization or infections in immune compromised hosts.

The publication of the whole genome sequence of the vancomycin resistant *E. faecalis* strain V583 (for genome data see http://www.tigr.org/) (192) enables us to study the genomics of the pathogenesis of *E. faecalis* infections in more detail. Construction of a DNA microarray containing all known genes of *E. faecalis* will make it possible to analyze the expression of genes in different environments (47, 187, 253). In analogue with the abundant presence of *E. faecalis* in the gastrointestinal tract and the rapid colonization of the gastrointestinal tract with nosocomial strains, it will be interesting to study the

Table 1. Suggested classification of the known virulence factors related to the stages of pathogenesis

<table>
<thead>
<tr>
<th>Virulence factors involved in</th>
<th>Virulence factor of <em>E. faecalis</em>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>nosocomial colonization</td>
<td>Esp, Agg</td>
</tr>
<tr>
<td>adhesion to cellular or abiotic surfaces</td>
<td>Esp, Agg, Ace</td>
</tr>
<tr>
<td>invasion or internalization of human tissue</td>
<td>Agg, cyt</td>
</tr>
<tr>
<td>tissue destruction</td>
<td>cyt, gel</td>
</tr>
<tr>
<td>immune evasion</td>
<td>escape from cellular immunity</td>
</tr>
<tr>
<td>immune evasion</td>
<td>escape from humoral immunity</td>
</tr>
<tr>
<td></td>
<td>Agg, epa, cyt</td>
</tr>
<tr>
<td></td>
<td>Esp</td>
</tr>
</tbody>
</table>

<sup>a</sup> Esp, enterococcal surface protein; Agg, aggregation substance; Ace, Adhesin to collagen; epa, enterococcal polysaccharide antigen; cyt, cytolysin; gel, gelatinase
expression of genes upon contact with intestinal epithelial cell lines in vitro. Furthermore, it might be interesting to study the influence of fecal components on the expression of different genes as the bacteria in the intestine possibly have more contact with feces than with the epithelial lining of the gut. These experiments might be expanded to the analysis of interaction with gelatinase and serine protease other host cells and human secretions that are frequently involved in enterococcal infections e.g. uroepithelial cells, urine or bile. Furthermore, the host response to enterococcal infections can be analyzed by DNA microarray analysis of the host cell lines. Comparison of the response of the host cell lines to isogenic strains of *E. faecalis* lacking particular virulence factors might also gain further insight in the host-pathogen interaction. These experiments might result in the definition of new putative virulence factors.

The next step in the analysis of new virulence factors will be to prove that the gene is truly associated with virulence and/or colonization. This can be done in different ways: (i) the construction of mutants lacking the gene under study and using this mutant in an animal infection model, (ii) prove of an immunological response to the microorganism and that specific immunity against the microorganism can protect against the infection and (iii) by comparative genetics, showing that the gene encoding the virulence factor is similar to known virulence factors in other species (251). However, all three methods might be difficult for virulence factors that are not immediately correlated with pathogenicity but with colonization and infection in immune compromised hosts by “commensal pathogens”. (i) The problem with the generation of mutants is that the mutant often also will be attenuated in its virulence if the gene is necessary for house keeping of the bacterium. Furthermore, if the gene under study is involved in the colonization of e.g. the human gastrointestinal tract this will be difficult to prove in an animal infection model. (ii) Factors that enhance the colonization of the host most likely will not elicit an immune response, and (iii) comparative genetics for genes that are associated with colonization can be difficult as not many genes are defined yet as colonization genes.

Other approaches to verify that a gene is necessary for survival and growth under certain conditions are genetic footprinting or signature-tagged mutagenesis. Both methods involve scanning of the genome by exhaustive transposon mutagenesis and subsequently screening the mutants en masse for functional properties (253). Comparison of pools of mutants grown under different conditions e.g. in broth culture and in the presence of fecal extract will lead to the identification of genes that are essential for the growth in certain environments because these mutants are not present in the pool grown under these conditions. This method can also be used to identify new virulence genes.

One drawback of the use of microarrays to study pathogenesis is the fact that microarrays can not measure expression of genes that are absent from the reference strain used to construct the microarray. Therefore, microarray analysis always needs to be completed with the search for new unknown genes. The identification of different genotypes associated with specific hosts or environments ([Chapter 2](#)) and one clone that spread between many patients ([Chapter 3](#)) can support this analysis of new virulence traits. In addition to this, new genogroups of *E. faecalis* can be identified by AFLP or multilocus sequence typing (MLST) of isolates from different patient groups and healthy volunteers from different geographical regions. Furthermore, new epidemic clones of enterococci that spread between patients can
be recognized by analyzing enterococci from different clinical samples with molecular epidemiological methods. Representatives from the new and known genogroups and epidemic clones can be analyzed in the search for new virulence factors. The analysis and comparison of the genomes of selected *E. faecalis* isolates in the search for new virulence factors can be done with different methods: (i) the different AFLP patterns can be compared and then DNA sequence of the differing bands can be analyzed. (ii) Another possibility is random sequencing of the genomes and searching for sequences encoding e.g. a surface protein. (iii) A sophisticated method of comparing genomes and identifying genes that are present in one genome but absent from another is subtractive hybridization (263). (iv) The ultimate method is sequencing of the different isolates and direct comparison of the genomes. The role in virulence or colonization of the genes identified with these methods should be analyzed taking into account that these genes might not be directly virulent in an animal model.

At the moment, infections with *E. faecalis* seem to be mainly restricted to immune compromised and severely ill patients. However, the emerging resistance of *E. faecalis* and the increasing number of infections in hospitalized patients might lead to a point where not only immune compromised patients get infected with this microorganisms but also persons with a healthy immune system. The dominant presence of *E. faecalis* might give it the opportunity to acquire virulence factors from other pathogens and combine them with its own virulence factors into one strain. If a strain subsequently also acquires resistance determinants against many antibiotics this will finally result in a “superbug” that is able to infect the normal healthy host and in addition is difficult to treat. The fact that this scenario is very well possible, is given by reports about the transfer of vancomycin resistance between strains (129) and the presence of components of the sex-pheromone plasmid transfer system in *Staphylococcus aureus*.

Therefore, it is important to keep on searching for the mechanisms of virulence and the Achilles heel of *E. faecalis* that can be used to treat and prevent infections with this microorganism.