The ecology, epidemiology and virulence of Enterococcus

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Enterococci are Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, which usually inhabit the alimentary tract of humans in addition to being isolated from environmental and animal sources. They are able to survive a range of stresses and hostile environments, including those of extreme temperature (5–65 °C), pH (4.5–10.0) and high NaCl concentration, enabling them to colonize a wide range of niches. Virulence factors of enterococci include the extracellular protein Esp and aggregation substances (Agg), both of which aid in colonization of the host. The nosocomial pathogenicity of enterococci has emerged in recent years, as well as increasing resistance to glycopeptide antibiotics. Understanding the ecology, epidemiology and virulence of Enterococcus species is important for limiting urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteremia and neonatal sepsis, and also stemming the further development of antibiotic resistance.

Introduction

For many years Enterococcus species were believed to be harmless to humans and considered unimportant medically. Because they produce bacteriocins, Enterococcus species have been used widely over the last decade in the food industry as probiotics or as starter cultures (Foulquie Moreno et al., 2006). Recently, enterococci have become one of the most common nosocomial pathogens, with patients having a high mortality rate of up to 61% (De Fátima Silva Lopes et al., 2005). In 2005 there were 7066 reported cases of bacteremia caused by Enterococcus species in the UK, an 8% increase from 2004, with the Health Protection Agency (2007) stating that ‘an increase in a bacteremia causing pathogen like this has not been observed for some time’. Twenty-eight per cent of all cases were antibiotic resistant (Health Protection Agency, 2007). The risk of death from vancomycin-resistant enterococci (VRE) is 75%, compared with 45% for those infected with a susceptible strain (Bearman & Wenzel, 2005). These figures are mirrored in the USA. Over a 15 year period there was a 20-fold increase in VRE associated with nosocomial infections reported to CDC’s National Nosocomial Infections Surveillance (NNIS) (National Nosocomial Infections Surveillance, 2004).

This dramatic increase in antibiotic resistance of Enterococcus species worldwide highlights the need for a greater understanding of this genus, including its ecology, epidemiology and virulence.

Taxonomy

The genus Enterococcus consists of Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. Enterococci belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins (Health Protection Agency, 2005). The genera of LAB with which Enterococcus are grouped are identified by a low G+C content of <50 mol% (Klein et al., 1998). There are no phenotypic characteristics to distinguish Enterococcus species from other Gram-positive, catalase-negative cocci bacteria, so identification is usually established by reverse methodology (elimination of other species traits first). As a genus Enterococcus has been recognized since 1899, when Thiercelin identified it as an intestinal organism (Stiles & Holzapfel, 1997); its taxonomy and ecology were reviewed by Klein (2003). Many attempts have been made to distinguish Enterococcus species from Streptococcus species. In 1937, Sherman classified Streptococcus species into four subgroups: faecal streptococci (enterococci), dairy streptococci, viridans group and pyogenous streptococci (Klein, 2003). Sherman noted that the enterococci subgroup included the Lancefield group D streptococci and suggested that the latter could be differentiated by haemolytic and proteolytic reactions, although this is inappropriate as haemolysis is determined by a plasmid (Stiles & Holzapfel, 1997). Traditional methods such as biotyping, serotyping and phage typing left questions as to which of the Streptococcus species actually belonged to the genus Enterococcus (Saeedi et al., 2002).
In 1984, through the use of DNA hybridization and 16S rRNA sequencing, it was established that the species *Streptococcus faecium* and *Streptococcus faecalis* were sufficiently distinct from the other streptococci to be designated another genus: *Enterococcus* (Foulquie Moreno et al., 2006). This means that the D group antigen is found in both streptococci and enterococci. Nine species were transferred from the *Streptococcus* groups and now *Enterococcus* includes 28 species (Foulquie Moreno et al., 2006). The molecular data that were collected using 16S rRNA sequencing of *Streptococcus* enabled the construction of an 16S rRNA-dendrogram showing the relationship between *Streptococcus*, *Enterococcus* and *Lactococcus* species (Fig. 1). This method also allowed the grouping of *Enterococcus* species. The *Enterococcus faecalis* species group includes *E. faecalis*, *Enterococcus haemoperoxidus* and *Enterococcus moraviensis* whilst the *Enterococcus faecium* species group includes *E. faecium*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus munditii*, *Enterococcus porcinus* and *Enterococcus vilorum* (Klein, 2003). The discrimination of enterococci from streptococci is mainly established by Lancefield group D antigen, as only *Streptococcus bovis*, *Streptococcus lactolyticus* and *Streptococcus equinus* are serogroup D. These groups can be distinguished from *Enterococcus* species by the lack of growth in 6.5% (w/v) sodium chloride at 10 °C. It is harder to distinguish *Enterococcus* species from other cocci that do not express the D group antigen such as *Pediococcus*, *Lactococcus* or *Tetragenococcus* species because no other phenotypic differences have been reported that allow distinction. Thus the use of fermentation patterns, enzyme activities such as pyroglutamyl aminopeptidase (PYRase) (Domig et al., 2003), growth at defined temperatures and physiological characteristics is essential in the identification of *Enterococcus* species (Shanks et al., 2006).

The differences in the genomes of *E. faecalis* and *E. faecium* were assessed in a study using competitive DNA hybridization (Shanks et al., 2006). *E. faecalis*-specific sequences compared with those of *E. faecium* mainly encoded surface-exposed proteins. Overall 6.4% of the *Enterococcus* genome is associated with cell-surface proteins and 22.6% of the differences between the two species are found in these genes. This variation is thought to have implications in the species avoiding different host immune responses (Shanks et al. 2006).

**Physiology**

*Enterococcus* species will grow at a range of temperatures from 5 to 50 °C. The optimum, minimum and maximum temperatures, according to the Rosso model, are 42.7, 6.5 and 47.8 °C, respectively, on brain heart infusion (BHI) agar in aerobic conditions (Van den Berghe et al., 2006), although growth will also occur in anaerobic atmospheres (Domig et al., 2003). Both *E. faecalis* and *E. faecium* can survive heating at 60 °C for 30 min, making *Enterococcus* species distinguishable from other closely related genera such as *Streptococcus* (Foulquie Moreno et al., 2006). Trypticase soy agar or Columbia agar with 5% (v/v) defibrinated sheep blood may be used to assess the haemolysis produced by enterococci. If human or horse blood is used, haemolysis is based on cytolysin activity and causes a β-haemolytic reaction (Domig et al., 2003). *E. faecalis* and *E. faecium* will grow in a wide range of pH (4.6–9.9), with the optimum being 7.5 (Van den Berghe et al., 2006). They will also tolerate and grow in the presence of 40% (w/v) bile salts. *E. faecalis* is able to grow in 6.5% NaCl and has a cation homeostasis which is thought to contribute to its resistance to pH, salt, metals and desiccation.

When assessing growth of *Enterococcus* species using optical densities the most important variable of the growth conditions is pH, with temperature and salt concentration having a lesser effect (Gardin et al., 2001). During the lag phase, temperature is the most important factor influencing growth, with stationary-phase cells being the most resistant to heat (Gardin et al., 2001; Martinez et al., 2003). The resistance of *E. faecalis* to a range of pH values is thought to be due to its membrane durability and impermeability to acid and alkali, although some studies have suggested that it may be associated with membrane-bound H⁺-ATPase activity (Nakajo et al., 2005). Temperature resistance is also associated with membrane structure and has been related to lipid and fatty acid content. The membrane has been demonstrated to be more stable near the minimal temperature for growth, which is a specific mechanism associated with enterococci (Ivanov et al., 2006).
al., 1999). At higher temperatures enterococci are less resilient, with the membrane fatty acid content increasing and the saturated fatty acid levels decreasing. The heat resistance of enterococci is dependent not only on the temperature but also the phase of growth (Martinez et al., 2003).

When *E. faecalis* is grown at non-stress temperatures, subsequently cultured cells do not have the resilience to warm and cold environments that would occur if the first generation were grown at stressful temperatures (Ivanov et al., 1999). Three distinct temperature groups (10–13 °C, 17–22 °C and 42–47 °C) have been established for *E. faecalis* at which permeability of the membrane to 3% NaCl is different. This has significant implications with regard to biotechnology and food science (Ivanov et al., 1999).

The production of amines is also closely related to the growth temperature and pH. The production of decarboxylases is optimum at acid pH, whereas biogenic amine production by *E. faecalis* EF37 decreases at low pH. Temperature does not have a significant effect on amine production itself, but the effect that temperature has on cell yield alters the quantity of amines being produced (Gardin et al., 2001). Other products of *Enterococcus* species that are affected by pH are bacteriocins.

### Bacteriocins

Bacteriocins are ribosomally synthesized, extracellular released antimicrobial peptides that show activity against closely related bacterial species. Four genes are required to produce bacteriocins: a structural gene encoding a prepeptide, a dedicated immunity gene, a dedicated ABC-immunity gene and a gene encoding the protein necessary for export of the bacteriocin. Bacteriocins are formed as prepeptides in the cell and mature during export from the cell. This occurs by the enzymic removal of an N-terminal leader peptide at a double glycine cleavage site, and export via a Sec-dependent pathway. Bacteriocins are cationic, amphiphilic proteins containing little or no cysteine, and their structures usually occur as random coils under aqueous conditions (Garneau et al., 2002). Bacteriocin production is favoured in stressful growth conditions, which is thought to be due to lower growth rates, resulting in better utilization of energy and greater availability of metabolites for the synthesis of bacteriocins. Under optimal growth conditions and thus high growth rates there is a lack of amino acids available for bacteriocin production (Van den Bergh et al., 2006). *Enterococcus* species are known to produce a range of enterocins (Table 1) including enterocins A, B, I, L and P, which are active against *Listeria* species, *Clostridium* species and *Staphylococcus aureus* (Campos et al., 2006). Most of the bacteriocins produced by *E. faecalis* and *E. faecium* are identical to enterocins A and B first described from *E. faecium* CTC492 and *E. faecium* T136 (De Kwaadsteniet et al., 2005). *E. faecium* RZS C5 is a natural cheese isolate, which is lacking in virulence factors and has antilisterial properties (Leroy et al., 2003). Enterocin EJ97 from *E. faecalis* S-47 has also shown antilisterial properties, with 1.6 log₁₀ reduction after 6 h and complete inhibition after 24 h at a concentration of 20 AU ml⁻¹ (Garcia et al., 2004). The enterocin AS-48 (produced by *E. faecalis* A-48-32) inhibits growth of *Bacillus coagulans* vegetative cells, at refrigeration and high temperatures, in canned fruits and vegetables (Lucas et al., 2006). The bacteriocin activity against Gram-negative bacteria is unusual, but bacteriocin ST15 from *E. mundtii* has been shown to be effective against a range of Gram-positive and Gram-negative bacteria including *Acinetobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Lactobacillus* and *Pseudomonas* (De Kwaadsteniet et al., 2005). The bactericidal effects of bacteriocins are thought to be

### Table 1. Bacteriocins produced by *Enterococcus* species

Adapted from De Kwaadsteniet et al. (2005).

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Produced by</th>
<th>Isolated from</th>
<th>Size (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterocins A and B</td>
<td><em>E. faecium</em> P21</td>
<td>Chorizo</td>
<td></td>
</tr>
<tr>
<td>Enterocin EJ97</td>
<td><em>E. faecalis</em> S-47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No name</td>
<td><em>E. faecium</em> A2000</td>
<td>Cheese</td>
<td></td>
</tr>
<tr>
<td>Enterocin CRL35</td>
<td><em>E. faecium</em> CRL35</td>
<td>Cheese</td>
<td>3500</td>
</tr>
<tr>
<td>Bacteriocin N15</td>
<td><em>E. faecium</em> N15</td>
<td>Nuka</td>
<td>3000–5000</td>
</tr>
<tr>
<td>Enterocins A and B</td>
<td><em>E. faecium</em> WEHE81</td>
<td>Cheese</td>
<td>4833 and 5462</td>
</tr>
<tr>
<td>No name</td>
<td><em>E. faecium</em> RZS C5; <em>E. faecium</em> DPC 1146</td>
<td>Porcine intestinal tract</td>
<td></td>
</tr>
<tr>
<td>AS-48</td>
<td><em>E. faecalis</em> subsp. <em>liquefaciens</em> S-48</td>
<td>Duodenum of ostrich</td>
<td>3400</td>
</tr>
<tr>
<td>Enterocin 012</td>
<td><em>E. gallinarum</em></td>
<td>Free-range chicken</td>
<td></td>
</tr>
<tr>
<td>No name</td>
<td><em>E. faecium</em> CRL 1385</td>
<td>Dry-fermented sausage</td>
<td></td>
</tr>
<tr>
<td>Enterocin P</td>
<td><em>E. faecium</em> P13</td>
<td>Facces of minipigs</td>
<td>4285 and 3899</td>
</tr>
<tr>
<td>Enterocins 1071A and 1071B</td>
<td><em>E. faecalis</em> BFE 1071</td>
<td>Vegetables</td>
<td>4287</td>
</tr>
<tr>
<td>Mundticin ATO6</td>
<td><em>E. mundtii</em> ATO6</td>
<td>Grass silage</td>
<td>4290</td>
</tr>
<tr>
<td>Mundticin KS</td>
<td><em>E. mundtii</em> NFRI 7393</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
due to permeabilization of the cell membrane. The random-coiled peptides of the bacteriocins, on contact with the cell membrane, form a helical structure, which incorporates into and spans the membrane, creating a pore. This mode of action has been observed in enterocin P. The resulting pore causes leakage of K⁺ ions, dissipation of membrane potential and inhibition of amino acid uptake. The cycle of ATP-driven K⁺ uptake by the cell and bacteriocin-mediated release of K⁺ leads to cell death (Garneau et al., 2002; Hechard & Sahl, 2002).

Ecology and epidemiology

The origins of Enterococcus species vary from environmental to animal and human sources. As enterococci are an essential part of the microflora of both humans and animals their distribution is very similar in these sources. E. faecium and E. faecalis are the most common in the human gastrointestinal tract, E. faecium in production animals and E. mundtii and E. casseliflavus in plant sources (Klein, 2003). The numbers of E. faecalis in human faeces range from 10⁵ to 10⁷ per gram, and those of E. faecium from 10⁴ to 10⁶ per gram. The isolation of E. faecium and E. faecalis is less prevalent from livestock than from human faeces (Franz et al., 1999).

Studies of the ecology and epidemiology of Enterococcus have reported E. faecalis and E. faecium being regularly isolated from cheese, fish, sausages, minced beef and pork (Foulquie Moreno et al., 2006; Klein, 2003). Foods such as sausages and cheese that are of animal origin are often associated with contamination by Enterococcus species, as they are able to survive the heating process. In one study in the UK, samples taken from urban sewage and from farmland using pig manure and crops generated from this land, were found to be 100 % positive for Enterococcus species. In crops to which animal fertilizers were not applied, the incidence of Enterococcus species was reduced to 33 % (Kuhn et al., 2003). A similar study in Germany isolated 416 strains of Enterococcus from 155 samples of food of animal origin, 72 % of which were E. faecalis and 13 % E. faecium (Peters et al., 2003).

The distribution of Enterococcus species varies throughout Europe. In Spain and the UK, E. faecalis and E. faecium are the most commonly isolated species from both clinical and environmental sources. Sweden has a lower incidence of E. faecium and a higher isolation rate of E. hirae, whereas in Denmark E. hirae is the dominant species and is isolated mainly from slaughtered animals (Kuhn et al., 2003).

Clinical isolates of enterococci show a lower diversity than those obtained from the environment and other human sources, with E. faecalis being the dominant species (Kuhn et al., 2003). The reason for this lack of diversity may be linked with the virulence factors associated with this species. The fact that Enterococcus species are opportunistic pathogens was highlighted by a study in Denmark which showed that hospitalized patients have a 57 % isolation rate of E. faecalis whereas healthy individuals show only a 39–40 % occurrence (Mutnick et al., 2003). Hospitalized patients may have a greater incidence of enterococcal infection not only because of virulence, but because the hospital itself is a hub. This is illustrated by a report for the Department of Health in the UK, which highlighted the fact that enterococci may contaminate and survive around the patient for several days (Brown et al., 2006). Enterococci also play a role in endodontic failure and are often isolated from the root canal system. The results of one study showed that out of 100 root-filled teeth with apical periodontitis, 69 % of the isolated bacteria were facultative and 50 % of those were enterococci (Dahlen et al., 2000). E. faecalis is responsible for 80–90 % of human enterococcal endodontic infection and is usually the only Enterococcus species isolated from the obturated root canal (Love, 2001; Peciuliene et al., 2001).

Rates of infection

Enterococcal infections include urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis (Poh et al., 2006). In Europe, infection with Enterococcus species was considered harmless to humans for a long time. However in the last decade enterococci have been reported as the second most common cause of wound and urinary tract infection and the third most common cause of bacteraemia (De Fátima Silva Lopes et al., 2005). In 2005 in the UK there were 7066 reported cases of Enterococcus bacteraemia, 63 % of these cases being due to E. faecalis and 28 % to E. faecium, both of which have increasing antibiotic resistance (Health Protection Agency, 2007). In the USA approximately 12 % of the hospital-acquired infections are Enterococcus species. E. faecalis is the most common species associated with clinical infection while E. faecium poses the higher antibiotic resistance threat (Giraffa, 2002).

Antibiotic resistance

The antibiotic resistance of Enterococcus is well documented. Bacteria may show resistance to glycopeptides such as vancomycin and teicoplanin, which are licensed in the UK, and to aminoglycosides (Kacmaz & Aksoy, 2005). Antibiotic resistance has been of growing concern for a number of years. Vancomycin was first used in the clinical arena in 1972 and the first vancomycin-resistant enterococci were recognized only 15 years later. NNIS reported an increase of 7.6 % in VRE between 1989 and 1993 (Metan et al., 2005). It has been reported that if glycopeptide-resistant enterococci (GRE) are present in an infected patient rather than an antibiotic-susceptible strain, clinical treatment failure is increased by 20 % and mortality is increased from 27 % to 52 % (Brown et al., 2006). When assessing the studies on enterococcal antibiotic resistance, the pattern that is emerging is the possible occurrence of multidrug resistant strains (Peters et al., 2003).
In both the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) and SENTRY (Antimicrobial Resistance Surveillance Program) databases, figures show that, of enterococcal isolates from the bloodstream, 2% of *E. faecalis* and 60% of *E. faecium* isolates are resistant to vancomycin (Bearman & Wenzel, 2005). Resistance rates of *Enterococcus* species have reached endemic or epidemic proportions in North America, with Europe having lower, but increasing, levels (Mutnick et al., 2003). Enterococcal antibiotic resistance is not exclusive to the clinical arena but is also prevalent in the food industry. The presence of VRE in individuals who have been hospitalized, when they have not previously been in hospital or taken antibiotics, suggests that VRE may have been contracted through the food chain. GRE may emerge in the food chain through use of avoparcin in animal feed (Mannu et al., 2003).

Glycopeptide resistance in enterococci involves a two-component system where the cell wall composition is altered from the peptidoglycan precursors D-Ala-D-Ala (vancomycin-susceptible) to D-Ala-D-lactate (D-Lac). The latter has 1000 times less affinity for vancomycin, while D-Ala-D-Ser has a sevenfold decrease in affinity for vancomycin, thus removing the susceptible target (Gilmore, 2002). The genes involved in this two-component system are *vanS/vanR*. The VanS sensor kinase is activated in response to vancomycin, resulting in the activation of D-Lac or D-Ser peptidoglycan precursor and the repression of D-Ala-D-Ala (Stephenson & Hoch, 2002). To date six gene clusters associated with glycopeptide resistance have been identified in *Enterococcus* species: *vanA* to *vanG* (Table 2). The three main types of resistance are those encoded by the vanC, vanA and vanB clusters. Intrinsic vanC resistance is specific to *E. gallinarum*, *E. casseliflavus* and *E. flavescens*, and the vanC operon is chromosomally located and is not transferable. The *vanA* resistance operon comprises seven genes (*vanH*, *vanA*, *vanX*, *vanR*, *vanS*, *vanY* and *vanZ*) and is acquired through the Tn1546 transposon (Gilmore, 2002). Over 100 enterococcal isolates from humans, animals and foods have shown *vanA* resistance residing on Tn1546 (Williams & Hergenrother, 2008). The transfer of *vanB* (acquired) resistance occurs through the exchange of transposon Tn1547 and/or Tn5382. Both *vanA* and *vanB* are present on the chromosome but can also be carried on a plasmid (Gilmore, 2002; Klare et al., 2003). *Enterococcus* species do not possess cytochrome enzymes and thus cannot produce the energy required to take up antibiotics into the cell. This means they show resistance to aminoglycosides at low levels (Klare et al., 2003). Antibiotic resistance in *Enterococcus* species can be transferred by pheromone-mediated conjugative plasmids or transposons. The resistance genes may be passed on not only to antibiotic-susceptible enterococci, but also to other pathogens (Giraffa, 2002).

In contrast to Gram-negative conjugation systems, conjugation of *Enterococcus* species does not require pili, and involves a pheromone-induced system (Andrup & Andersen, 1999). Bacteria containing conjugative plasmids respond to pheromones (plasmid specific) for genetic exchange; these bacteria generally have a narrow recipient range for conjugation, including only closely related species. This lateral transfer of genetic elements leads to rapid dissemination of antibiotic resistance. The plasmids occurring in *Enterococcus* species can also be transmission vehicles for transposons (Williams & Hergenrother, 2008). The most extensively investigated pheromone-inducible plasmids in *E. faecalis* are pCF10, pAD1 and pPD1. In the case of pAD1 the trans-acting regulatory protein encoded by the *traE* gene is expressed (Folli et al., 2008). The transfer of these plasmids occurs in response to specific sex pheromone peptides secreted by plasmid-free recipient cells. Uptake of the exogenous pheromone by the donor cell causes it to express proteins involved in the conjugation process. Production of aggregation substance (Agg) on the donor cell surface facilitates contact with the recipient cell by binding to enterococcal binding substance (EBS) displayed on the surface, resulting in conjugation and the ability to pass antibiotic resistance on to the recipient cell (Clewell et al., 2002). The pAD1 plasmid has also been shown to carry the Tn917 transposon associated with *E. faecalis*; conjugal transfer of Tn916 involves excision of a circular intermediate that is transferred via a plasmid into the recipient cell where it inserts into the recipient chromosome (Gilmore, 2002). Pheromones released for plasmids pCF10, pAD1 and pPD1 are pAD1 or cCF10, cAD1 and cPD1 respectively (Folli et al., 2008).

### Table 2. Vancomycin resistance genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vancomycin MIC (µg ml⁻¹)</th>
<th>Location</th>
<th>Expression</th>
<th>Precursor</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>64–1000</td>
<td>Plasmid or chromosome</td>
<td>Inducible</td>
<td>D-Ala-D-Lac</td>
</tr>
<tr>
<td>vanB</td>
<td>4–1000</td>
<td>Plasmid or chromosome</td>
<td>Inducible</td>
<td>D-Ala-D-Lac</td>
</tr>
<tr>
<td>vanC</td>
<td>2–32</td>
<td>Chromosome</td>
<td>Constitutive or inducible</td>
<td>D-Ala-D-Lac</td>
</tr>
<tr>
<td>vanD</td>
<td>64–168</td>
<td>Chromosome</td>
<td>Constitutive</td>
<td>D-Ala-D-Lac</td>
</tr>
<tr>
<td>vanE</td>
<td>16</td>
<td>?</td>
<td>Inducible</td>
<td>D-Ala-D-Ser</td>
</tr>
<tr>
<td>vanG</td>
<td>&lt;16</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

Adapted from Gilmore (2002).
In *E. faecalis* the proteins RepA, RepB and RepC and the *par* locus are involved in the regulation of the pheromone-responding pAD1 replicon. The *repA* gene encodes a replication initiator protein, while *repB* and *repC* are involved in control of the replication frequency and stability of the plasmid (Weaver *et al.*, 2009). *Enterococcus* plasmids can also be utilized for the genetic exchange of virulence factors.

**Virulence**

*Enterococcus* species with the highest virulence are medical isolates, followed by food isolates and then starter strains (Busani *et al.*, 2004; Ben Omar *et al.*, 2004). Many factors determine the virulence of *Enterococcus* species, for example (1) ability to colonize the gastrointestinal tract, which is the normal habitat; (2) ability to adhere to a range of extracellular matrix proteins, including thrombospondin, lactoferrin and vitronectin; and (3) ability to adhere to urinary tract epithelia, oral cavity epithelia and human embryo kidney cells. Most infection is thought to be endogenous, by translocation of the bacteria through the epithelial cells of the intestine, which then cause infection via lymph nodes and thus spread to other cells within the body (Franz *et al.*, 1999). The aggregation substance (Agg) on the surface of *E. faecalis*, has been shown in *vivo* to form large aggregates and hence may contribute to pathogenesis. The presence of Agg increases the hydrophobicity of the enterococcal cell surface. This induces localization of cholesterol to the phagosomes and is thought to delay or prevent fusion with lysosomal vesicles (Eaton & Gasson, 2002). Agg is a pheromone-inducible surface glycoprotein and mediates aggregate formation during conjugation, thus aiding in plasmid transfer as well as adhesion to an array of eukaryotic surfaces (Koch *et al.*, 2004). Pulsed-field gel electrophoresis analysis of clinical isolates of *E. faecalis* showed that the gene encoding Agg was not present in *E. faecium* isolates (Hallgren *et al.*, 2008). Another cell-surface protein present in *E. faecalis* is Ace (adhesion of collagen from *E. faecalis*). This is a collagen-binding protein, belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family. Ace may play a role in the pathogenesis of endocarditis (Koch *et al.*, 2004).

Extracellular surface protein (Esp) is a cell-wall-associated protein first described in *Enterococcus* species by Shankar *et al.* (1999). The *esp* gene consists of 5622 bp and is found at high frequency in infection-derived isolates. It is thought to promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance (Foulquie Moreno *et al.*, 2006). Esp also contributes to enterococcal biofilm formation, which could lead to resistance to environmental stresses, and adhesion to eukaryotic cells such as those of the urinary tract (Borgmann *et al.*, 2004). Studies have shown that disruption of the *esp* gene impairs the ability of *E. faecalis* to form biofilms. Esp-negative *E. faecalis* strains, after receiving plasmid transfer of the *esp* gene, were able to produce biofilms (Latasa *et al.*, 2006). Twenty-one out of 28 clinical isolates of *E. faecium* were found to have sequences that were specific for the *esp* gene. This goes some way to suggesting that the *esp* gene may be associated with pathogenicity, since the *esp* gene was absent from dairy isolates (Mannu *et al.*, 2003). *E. faecium* strains that carry the gene *esp_m* have higher conjugation rates than strains that do not possess this gene. They also demonstrate higher resistance to ampicillin, ciprofloxacin and imipenem (Billstrom *et al.*, 2008).

The ability of enterococci to produce biofilms is fundamental in causing endodontic and urinary tract infections, as well as endocarditis. The formation of pili by enterococci is necessary for biofilm formation, the gene cluster associated with this being *ebp* (endocarditis- and biofilm-associated pili). The *ebp* operon consists of *ebpA*, *ebpB* *ebpC* and an associated *srtC* (encoding sortase C) gene (Singh *et al.*, 2007). A non-piliated mutant of *E. faecalis* was unable to produce a biofilm (Budzik & Schneewind, 2006). Enterococcal pili are heterotrimetric and the pilus shaft contains two minor pilins. A feature of Gram-positive pili is that a specific sortase is dedicated to their assembly (Mandlik *et al.*, 2008). The pili are constructed by cross-linking of multiple classes of precursor proteins that are assigned by sortases, which covalently anchor proteins with a C-terminal pilin-associated motif to the peptidoglycan (Nallapareddy *et al.*, 2006). *E. faecalis* contains two classes of sortase: sortase A links most proteins with a C-terminal sortase motif to cell wall peptidoglycan, while sortase C is designated Bps (biofilm and pilus-associated sortase) and links the pilin subunits.

Secreted virulence factors of *Enterococcus* species also have a function in pathogenesis. Cytolysin (also called haemolysin) is a bacterial toxin, the genes for the production of which are located on pheromone-responsive plasmids (Koch *et al.*, 2004). Cytolysin has β-haemolytic properties in humans and is bactericidal against other Gram-positive bacteria. The *cyILa* group of genes are the non-regulatory genes of the cytolsin operons (Hallgren *et al.*, 2008), and higher incidences of these genes occur in clinical isolates (33 %, compared to 6 % in food isolates) (Semedo *et al.*, 2003). Cytolysin is regulated by a quorum-sensing mechanism involving a two-component system.

A group of hydrolytic enzymes including hyaluronidases, gelatinase and serine protease are involved in the virulence of *Enterococcus* species, although their precise roles are yet to be clearly understood (Semedo *et al.*, 2003). Hyaluronidase acts on hyaluronic acid and is a degradative enzyme which is associated with tissue damage. Hyaluronidase depolymerizes the mucopolysaccharide moiety of connective tissue, thus facilitating spread of enterococci as well as their toxins through host tissue (Kayaoğlu & Orstavik, 2004). Hyaluronidase is encoded by the chromosomal *hyl* gene. One study showed that, out of 26 vancomycin-resistant *E. faecalis* clinical isolates, seven
(27 %) carried the hyl gene, but it was found in only 14 % of faecal isolates (Vankerckhoven et al., 2004).

The main role of both gelatinase and serine protease in enterococcal pathogenesis is thought to be in providing nutrients to the bacteria by degrading host tissue, although they also have some function in biofilm formation (Gilmore, 2002; Mohamed & Huang, 2007). Gelatinase (GeIE) is an extracellular zinc metallo-endopeptidase secreted by E. faecalis (Koch et al., 2004). It is able to hydrolyse gelatin, casein, haemoglobin and other bioactive peptides. The gene (gelE) encoding GeIE is located on the chromosome and is regulated in a cell-density-dependent manner. Another gene sprE, coding for a serine protease, is located directly downstream from and is cotranscribed with gelE (De Fátima Silva Lopes et al., 2006). Transcription of gelE and sprE is regulated in a growth-phase-dependent fashion by the quorum-sensing system encoded by the fsr (faecal streptococci regulator) locus (Sifri et al., 2002).

Quorum sensing occurs when a bacterial population produces a signal via an autoinducing peptide (AIP), regulated by a two-component system. AIP then accumulates in the environment by increased expression of the communication signal, or by increased numbers of cells producing the signal. Once the AIP reaches a threshold concentration, it interacts with a cell-surface receptor or re-enters the cell and causes a cascade of transcriptional regulation (Alksne & Projan, 2000; Gobbetti et al., 2007). The fsr locus contains the fsrA, fsrB and fsrC genes. The fsrA gene is monocistronically transcribed into a response regulator, and fsrB and fsrC, encoding a processing enzyme and a sensor kinase respectively, are co-transcribed (Podbielski & Kreikemeyer, 2004). FsrB liberates gelatinase biosynthesis activating pheromone (GBAP) peptide, and with the accumulation of GBAP a transition from exponential to stationary phase occurs and gelE and sprE are induced. It has been shown that in E. faecalis when mutations in fsrA, fsrB and fsrC are present, a reduction in biofilm formation of 28–32 % occurs (Mohamed & Huang, 2007). All of 12 E. faecalis endocarditis strains were positive for the fsr locus while 10 out of 19 stool strains had the fsr locus, indicating the importance of fsr in virulence and disease (Podbielski & Kreikemeyer, 2004).

**Conclusions**

Although an array of studies into the taxonomy and identification of Enterococcus species has been carried out in recent years (Saeedi et al., 2002; Foulquie Moreno et al., 2006; Klein, 2003), it is still relatively difficult to distinguish Enterococcus species from other LAB, particularly Streptococcus expressing group D antigen. It is also difficult to perform inter-species identification of E. faecium and E. faecalis (Klein, 2003), but recent developments in genetic typing have made this easier. The identification of Enterococcus species is vital because for many years enterococci were believed to be harmless to humans and considered unimportant medically. They were thought mainly to be part of the human endogenous non-pathogenic microflora (Franz et al., 1999). Recently, enterococci have become one of the most common nosocomial pathogens, giving a high mortality rate of up to 61 % (De Fátima Silva Lopes et al., 2005). The ability of Enterococcus species to survive a range of adverse environments (Van den Bergh et al., 2006) allows multiple routes of cross-contamination of enterococci in causing human disease, including those from food, environmental and hospital sources. Overall, greater understanding of the ability of Enterococcus species to survive stresses, of virulence traits, and especially of increasing antibiotic resistance, is needed in order to fully appreciate the complexity of Enterococcus species in causing disease.

**References**


