VIRULENCE FACTORS OF *ENTEROCOCCUS FAECALIS*: RELATIONSHIP TO ENDODONTIC DISEASE

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ABSTRACT: *Enterococcus faecalis* is a micro-organism that can survive extreme challenges. Its pathogenicity ranges from life-threatening diseases in compromised individuals to less severe conditions, such as infection of obturated root canals with chronic apical periodontitis. In the latter situation, the infecting organisms are partly shielded from the defense mechanisms of the body. In this article, we review the virulence factors of *E. faecalis* that may be related to endodontic infection and the periradicular inflammatory response. The most-cited virulence factors are aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin. Each of them may be associated with various stages of an endodontic infection as well as with periapical inflammation. While some products of the bacterium may be directly linked to damage of the periradicular tissues, a large part of the tissue damage is probably mediated by the host response to the bacterium and its products.

Key words: *Enterococcus faecalis*, virulence factors, endodontic infection, apical periodontitis.

Introduction

Enterococci inhabit the gastrointestinal tract, the oral cavity, and the vagina in humans as normal commensals. They can cause a wide variety of diseases in humans, infecting the urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds, and indwelling foreign devices (Jett *et al.*, 1994). Enterococci now rank among the top three nosocomial bacterial pathogens (Richards *et al.*, 2000; Wisplinghoff *et al.*, 2003), and strains resistant to currently available antibiotics pose real therapeutic difficulties (Hunt, 1998). Up to 90% of enterococcal infections in humans are caused by *Enterococcus faecalis*. The majority of the remainder is caused by *Enterococcus faecium*, and infections with the other species are quite rare (Jett *et al.*, 1994). Enterococci have also been implicated in endodontic infections. Although they make up only a small proportion of the initial flora of untreated teeth with necrotic pulps (Sundqvist, 1992), enterococci, particularly *E. faecalis*, have been frequently found in obturated root canals exhibiting signs of chronic apical periodontitis, isolated in 23-70% of the positive cultures (Engström, 1964; Möller, 1966; Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Peciuliene *et al.*, 2000; Hancock *et al.*, 2001) and often occur in monoculture (Sundqvist *et al.*, 1998; Dahlén *et al.*, 2000; Peciuliene *et al.*, 2000; Hancock *et al.*, 2001). Moreover, *E. faecalis* was among a group of bacteria cultured from periapical lesions refractory to endodontic treatment (Sunde *et al.*, 2002).

Enterococci can withstand harsh environmental conditions. As originally defined by Sherman (1937), enterococci can grow at 10°C and 45°C, at pH 9.6, in 6.5% NaCl broth, and survive at 60°C for 30 minutes. *E. faecalis* can adapt to adverse conditions: Following pre-exposure to sublethal stress conditions, *E. faecalis* becomes less sensitive to normally lethal levels of sodium dodecyl sulfate, bile salts, hyperosmolarity, heat, ethanol, hydrogen peroxide, acidity, and alkalinity; furthermore, 'cross-protection' is pronounced against diverse challenges (Flahaut *et al.*, 1996a,b,c, 1997). Starving *E. faecalis* cells maintain their viability for extended periods and become resistant to UV irradiation, heat, sodium hypochlorite, hydrogen peroxide, ethanol, and acid (Giard *et al.*, 1996; Hartke *et al.*, 1998). *E. faecalis*, moreover, can enter the viable but non-cultivable (VBNC) state, a survival mechanism adopted by a group of bacteria when exposed to environmental stress, and resuscitate upon returning to favorable conditions (Lleò *et al.*, 2001). The ability of *E. faecalis* to tolerate or adapt to harsh environmental conditions may act as an advantage over other species. It may explain its survival in root canal infections, where nutrients are scarce and there are limited means of escape from root canal medicaments.

In *in vitro* studies, *E. faecalis* has been shown to invade dentinal tubules (Akpata and Blechman, 1982; Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990; Love, 2001), whereas not all bacteria have this ability (Akpata and Blechman, 1982; Perez *et al.*, 1993). In animal studies, where pure cultures of various bacteria were inoculated separately into root canals, *E. faecalis*, unlike others, was found to colonize the root canal in most cases and to survive without the support of other bacteria (Fabricius *et al.*, 1982; Sobrinho *et al.*, 1998). *E. faecalis* is resistant to the antimicrobial effects of calcium hydroxide (Byström *et al.*, 1985; Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990; Distel *et al.*, 2002), probably partly due to an effective proton pump mechanism which maintains optimal cytoplasmic pH

levels (Evans *et al.*, 2002). Besides, *E. faecalis*, intrinsically or *via* acquisition, may be resistant to a wide range of antibiotics (Leclercq, 1997; Hunt, 1998), which, if used, may shift the microbial flora in favor of *E. faecalis*.

We undertook a literature search for the virulence factors of *E. faecalis*, which may relate to colonization of the host, competition with other bacteria, resistance against defense mechanisms of the host, and production of pathological changes directly through production of toxins or indirectly through induction of inflammation. The factors most extensively studied are: aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, and cytolysin (hemolysin). Although not strictly acting as virulence factors, AS-48 and other bacteriocins are mentioned because of their possible contribution to the dominance of *E. faecalis* in persistent endodontic infections. From the data available, a model for the pathogenicity of *E. faecalis* in endodontic infections has been developed (Fig.), where the elements of virulence factors and means of ecological advantage for this organism have been integrated.

Aggregation Substance

Aggregation substance (AS) is a pheromone-responsive, plasmid-encoded bacterial adhesin that mediates efficient contact between donor and recipient bacterium, facilitating plasmid exchange. While the AS is expressed by the donor cell, the bacterial conjugation process requires that 'binding substance' (BS, the chromosomally encoded cognate ligand for AS) be expressed on the surface of the recipient cell. A mutant strain of *E. faecalis* lacking BS failed to act as a recipient in broth matings yet retained recipient ability in filter membrane matings, where no AS-BS interaction was necessary (Trotter and Dunny, 1990). AS is proteinaceous, appears as a hair-like structure on the cell surface, and is incorporated into the 'older' parts of the cell wall (Wanner *et al.*, 1989). Its expression on the cell surface may be induced by serum (Kreft *et al.*, 1992)

In addition to its adhesive function during the bacterial conjugation process, AS mediates adhesion of *E. faecalis* to a variety of eukaryotic cells *in vitro*, including renal tubular cells (Kreft *et al.*, 1992) and intestinal epithelial cells (Olmsted *et al.*, 1994). The sequence analysis of the AS indicates two RGD motifs (arginine, glycine and aspartic acid, amino acids) that are thought to facilitate adherence of the bacterium to the host cell *via* integrins, a family of eukaryotic cell-surface receptors (Galli *et al.*, 1990). However, analysis of recent data suggests that a non-RGD-dependent pathway of Asc10 (a group of AS proteins) mediated cell internalization into enterocytes (Waters *et al.*, 2003).

AS was also found to mediate binding to extracellular matrix (ECM) proteins, including collagen type I. *E. faecalis* strain OG1X(pAM721), constitutively expressing AS, binds to collagen type I more than two times higher than the AS-negative strain OG1X(pAM944) (Rodzinski *et al.*, 2001). Binding to collagen type I by bacteria may be of particular importance with respect to endodontic infections, since this is the main organic component of the dentin (Linde and Goldberg, 1993). A novel representative of the AS family, Asa 373, differing in its protein structure from classic AS, was reported to exhibit some moderately conserved amino acid motifs when its database sequence was compared with those of some other bacterial adhesins. The greatest similarity was with the amino acid sequence of Ag I/II polypeptides of oral streptococci (Muscholl-Silberhorn, 1999). The Ag I/II family of polypeptides has been found to mediate collagen recognition in some oral streptococci, and this has been associated with their ability to invade dentin tubules (Love *et al.*, 1997). However, there is no direct evidence to support a role for Asa 373 to mediate binding to collagen.

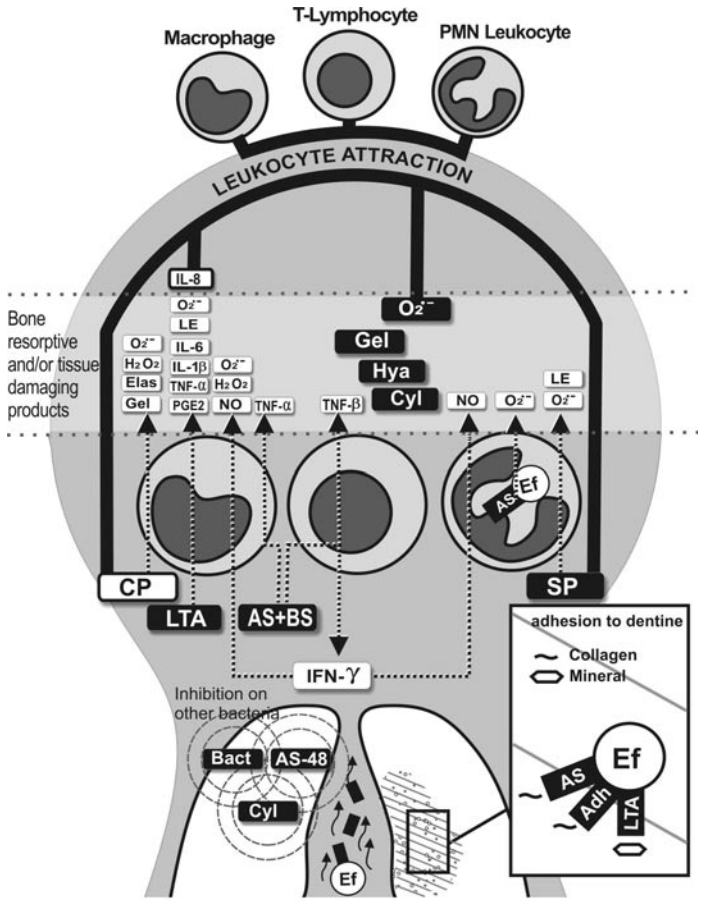

Figure. An endodontic disease model related to virulence factors of *E. faecalis*. The virulence factors of the bacterium inside the dentinal tubules and the root canal are released to the periradicular area, where they elicit leukocyte attraction or stimulate leukocytes to produce inflammatory mediators or lytic enzymes. Some of the bacteria may translocate to the periradicular lesion as well. The injurious virulence factors and leukocyte products are shown in the zone between the interrupted lines. In a magnified window, the adhesion of the bacterium to diverse elements of the dentin is depicted. Bacterial products fighting other bacteria are also included. Note that names in black boxes are the products of the bacterium. Abbreviations: Adh, surface adhesins; AS, aggregation substance; Bact, bacteriocins; BS, binding substance; CP, collagen peptides; Cyl, cytolysin; Ef, *Enterococcus faecalis*; Elas, elastase; Gel, gelatinase; Hya, hyaluronidase; H_2O_2, hydrogen peroxide; IFN-γ, gamma interferon; IL, interleukin; LE, lysosomal enzymes; LTA, lipoteichoic acid; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PGE2, prostaglandin E2; SP, sex pheromones; and TNF, tumor necrosis factor. $O_2^{\cdot-}$: The dot denotes the presence of an unpaired electron, and the superscript denotes the negative charge.

AS has been reported to promote direct, opsonin-independent binding of *E. faecalis* to human neutrophils *via* a complement receptor-mediated mechanism (Vanek *et al.*, 1999). As a consequence of this special type of binding, *E. faecalis*-bearing AS was shown to be resistant to killing by human neutrophils, despite marked phagocytosis and neutrophil activation (Rakita *et al.*, 1999). Furthermore, both PMN-mediated extracellular

superoxide production and phagosomal oxidant production against the AS-expressing strains were higher than those against the control strains lacking AS (Rakita *et al.*, 1999). This oxidative burst by the neutrophils may be a possible contribution to tissue damage in case of infection with cells of *E. faecalis* expressing AS (Fig.). AS was also reported to promote opsonin-independent adherence to and phagocytosis of *E. faecalis* by human macrophages as well, facilitating intracellular survival time in macrophages. However, AS suppresses the respiratory burst triggered by macrophages, as indicated by reduced concentrations of superoxide anion (Süßmuth *et al.*, 2000). The responses to the AS-expressing *E. faecalis* by human neutrophils and macrophages, therefore, appear to vary; however, it can be concluded that AS serves as a protective factor in favor of the bacterium against the host defense mechanisms.

Superantigens are molecules produced by bacteria, viruses, parasites, and yeasts which can induce inflammation through stimulation of T-lymphocytes, followed by massive release of inflammatory cytokines, resulting in tissue damage (Jappe, 2000). AS, in combination with BS, was reported to possess superantigen activity (Schlievert *et al.*, 1998). Cell extracts of AS- and BS-positive *E. faecalis* were found to induce T-cell proliferation, with subsequent release of tumor necrosis factor beta (TNF-β) and gamma interferon (IFN-γ), and to activate macrophages to release tumor necrosis factor alpha (TNF-α) (Fig.). The stimulation of lymphocyte proliferation and the production of the cytokines were comparable with those occurring following stimulation with the established staphylococcal superantigen toxic shock syndrome toxin-1 (TSST-1), as the positive control (Schlievert *et al.*, 1998). The cytokines TNF-α and TNF-β have been implicated in bone resorption (Stashenko, 1998), while IFN-γ has been considered as an irreplaceable factor in host defense against infection and, at the same time, as an inflammatory mediator (Billiau, 1996). IFN-γ is well-known to potentiate respiratory burst responsiveness of macrophages to stimulants, resulting in increased production of hydrogen peroxide and superoxide anion. IFN-γ also stimulates the production of the cytotoxic agent nitric oxide (NO) by a variety of cells, including macrophages and neutrophils (Fig.), and may cause undesirable cell and tissue damage.

Results from animal studies concerning the role of AS in *E. faecalis* pathogenesis vary. In studies involving rabbits, AS promoted endocarditis (Chow *et al.*, 1993; Schlievert *et al.*, 1998), whereas this was not the case in a rat endocarditis model (Berti *et al.*, 1998), and AS did not affect the severity of the disease in a rabbit endophthalmitis model (Jett *et al.*, 1998). The issue of promotion of endocarditis by *E. faecalis* may be viewed in the general context of an interaction between a bacterial adhesin and a host target. The extracellular matrix of all mammalian tissues consists of glycoproteins (*e.g.*, collagen, laminin, fibronectin) and proteoglycans that can be exploited by micro-organisms for colonization and initiation of infection (Westerlund and Korhonen, 1993). The ability of a bacterium to adhere to collagen has been shown to play an important role in the pathogenesis of endocarditis (Hienz *et al.*, 1996). Since dentinal tissues (Linde and Goldberg, 1993) share common ECM proteins with the heart tissue (Bashey *et al.*, 1992), a role for AS in endocarditis may also have relevance for endodontic infections. In epidemiologic studies, AS has frequently been detected in clinical isolates (Ike *et al.*, 1987; Elsner *et al.*, 2000) but is rarely found among fecal isolates from healthy volunteers (Coque *et al.*, 1995), suggesting a possible role for AS in human enterococcal infections.

Surface Adhesins

Enterococcal gene *esp*, encoding the high-molecular-weight surface protein Esp, has been detected in abundance among bacteremia and endocarditis isolates, but is rare in stool isolates from healthy individuals (Shankar *et al.*, 1999; Archimbaud *et al.*, 2002). The contribution of the surface protein Esp to colonization and persistence of *E. faecalis* in urinary tract infections has been shown in an animal model (Shankar *et al.*, 2001). Esp is also associated with promotion of primary attachment and biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana *et al.*, 2001). Furthermore, biofilm formation by *E. faecalis* has also been observed on medicated dentinal walls, and this form of organization could allow the bacteria to resist the bactericidal effect of calcium hydroxide medication in infected root canals (Distel *et al.*, 2002).

The *efaA* gene was identified with the use of an antiserum from a patient with *E. faecalis* endocarditis (Lowe *et al.*, 1995). The amino acid sequence of the associated protein, EfaA, revealed 55 to 60% homology to a group of streptococcal proteins known as adhesins. Thus, it was hypothesized that EfaA might be functioning as an adhesin in endocarditis. Production of EfaA by strains of *E. faecalis* is common. In one study, the *efaA* gene was detected in all medical (blood, pus, urine, feces, hospital environment) and almost all food (milk, cheese, meat) isolates of *E. faecalis* (Eaton and Gasson, 2001). In an animal model, mutants with the *efaA* gene showed prolonged survival, compared with *E. faecalis* strains bearing no *efaA* gene, indicating a role for the *efaA* gene in disease (Singh *et al.*, 1998a). Recent studies suggest EfaA as a solute binding-protein receptor for a manganese transport system in *E. faecalis*. While manganese is required for the growth and survival of most micro-organisms, EfaA is strongly expressed in a manganese-ion-depleted environment, probably for the regulation of the cytoplasmic homeostasis of the cation (Low *et al.*, 2003). The relatively low availability of manganese in serum (Krachler *et al.*, 1999) and in dentin (Battistone *et al.*, 1967) may induce expression of EfaA *in vivo*.

Studies have also focused on factors associated with the binding of bacteria to extracellular matrix (ECM) proteins. Various strains of *E. faecalis* obtained from different clinical materials were found to agglutinate strongly with ECM proteins, including collagen type I and type IV, and this was attributed to the surface hydrophobicity of the cells (Zareba *et al.*, 1997). The study by Xiao *et al.* (1998) indicated that a protein was involved in the binding of *E. faecalis* to ECM proteins following growth in a stressful condition, defined as growth at 46°C. The so-called 'conditional adherence' of the bacterium to collagen, however, was impaired following pre-treatment with a protein-digesting enzyme or pre-incubation with soluble forms of collagen, or following digestion of the binding substrate with collagenase. The putative proteinaceous adhesin of *E. faecalis* was subsequently identified as Ace, a collagen-binding MSCRAMM (microbial surface component recognizing adhesive matrix molecules), which is structurally and functionally similar to the collagen-binding protein Cna of *Staphylococcus aureus* (Rich *et al.*, 1999). It was recently shown that the disruption of the *ace* gene impaired the conditional binding of *E. faecalis* to the ECM proteins (Nallapareddy *et al.*, 2000b). Identification of Ace-specific antibodies in sera obtained from patients with enterococcal infections, and especially from patients with *E. faecalis* endocarditis, indicated that Ace is commonly expressed *in vivo* during human infections by

different strains, and not just at 46°C *in vitro* (Nallapareddy *et al.*, 2000a). Recently, the influence of Ace on adhesion of the bacterium to dentin was tested (Hubble *et al.*, 2003). As compared with the Ace-negative isogenic strain, Ace producing wild-type strain OG1RF adhered significantly more to dentinal surfaces when both strains were incubated at 46°C. In addition, serine protease was found to aid adhesion of the bacterium to dentin, probably by exposing binding sites for the adhesins or by modifying the adhesins. Interestingly, adhesion of the Ace-positive strain to dentin at 37°C was far superior to adhesion at 46°C. This may be an indication of a stronger adhesin working at 37°C or an unidentified factor present in dentin (or in the culture medium) which enhances the expression of Ace at 37°C.

Several investigators have demonstrated that serum may modulate bacterial surface antigen expression (Guzman *et al.*, 1989, 1991; Lambert *et al.*, 1990; Lowe *et al.*, 1995). Guzman *et al.* (1989, 1991) suggested that adherence of *E. faecalis* to eukaryotic cells could be mediated by carbohydrate residues present on the bacterial cell surface. *E. faecalis* isolates from urinary tract infections (UTI) express D-galactose and L-fucose ligands when grown in serum, whereas the isolates normally did not express these ligands when they were grown in brain heart infusion broth (Guzman *et al.*, 1991). Growth in serum raised the adherence of *E. faecalis* isolates from UTI and endocarditis to eukaryotic cells by at least 1·5- to three-fold, with the greatest (eight-fold) increase in adherence of UTI strain to heart cells (Guzman *et al.*, 1989). A role for serum in invasion of dentinal tubules by *E. faecalis* was suggested by Love (2001). In the presence of human serum, dentin invasion and collagen adhesion by the other test species, *Streptococcus gordonii* DL1 and *Streptococcus mutans* NG8, was inhibited, while dentin invasion by *E. faecalis* JH2-2 was not inhibited, and binding to collagen was enhanced.

Sex Pheromones

Sex pheromones are chromosomally encoded, small, hydrophobic peptides, 7 or 8 amino acids long, which function as signaling peptides in *E. faecalis* (Clewell and Weaver, 1989). Production of the sex pheromones by strains of *E. faecalis* and its bacterial clumping-inducing effect was first documented in 1978 by Dunny and co-workers (Dunny *et al.*, 1978). The transfer frequency of certain conjugative plasmids in *E. faecalis* is known to be increased several-fold by the sex pheromone system. Briefly, the latter phenomenon occurs as follows. A recipient strain secretes into the medium the sex pheromone corresponding to the plasmid which it does not carry. In response, the donor strain produces the AS adhesin (see above) that provides tight contact between the recipient and the donor strain, facilitating the conjugative transfer of the replicated plasmid. Once a copy of the plasmid is acquired, the recipient shuts off the production of that pheromone, but continues to secrete pheromones specific for other plasmids that it does not carry. Antibiotic resistance and other virulence traits, such as cytolysin production, can be disseminated among strains of *E. faecalis via* the sex pheromone system (Clewell and Weaver, 1989).

Some of the *E. faecalis* sex pheromones and their inhibitory peptides were found to be chemotactic for human and rat neutrophils and also to induce superoxide production and lysosomal enzyme secretion (Ember and Hugli, 1989; Sannomiya *et al.*, 1990) (Fig.). Studies have demonstrated a strong association between gingival crevicular fluid neutrophil lysosomal enzymes and chronic periodontal disease (Kunimatsu *et al.*, 1995; Buchmann *et al.*, 2002). A phagocytic lysosomal enzyme, arylsulfatase, has been detected in abundance in periapical lesions, whereas samples from healthy tissues showed little or no enzyme activity (Aqrabawi *et al.*, 1993), and teeth with larger periapical lesions exhibit increased levels of the lysosomal enzyme beta glucoronidase (Kuo *et al.*, 1998). Furthermore, neutrophil lysosomal enzymes may activate the complement system, which can contribute to bone resorption in the periapical tissues either by destruction of bone or by inhibition of new bone formation (Torabinejad *et al.*, 1985).

Lipoteichoic Acid

Lipoteichoic acids (LTA) are a group of closely related amphipathic molecules consisting of a polyglycerolphosphate backbone (1-3 phosphodiester-linked chains of 25 to 30 glycerolphosphate residues variously substituted with glycosyl and D-alanyl ester groups) joined covalently to a glycolipid moiety. They are often present on the cell surfaces of many Gram-positive bacteria (Wicken and Knox, 1975) and also in the culture fluid of streptococci and lactobacilli (Markham *et al.*, 1975). Most of the oral streptococci have been shown, by immunoassay, to have polyglycerolphosphate-containing LTA (Hogg *et al.*, 1997).

Through its lipidic moiety, the LTA molecule has been found to bind to a variety of eukaryotic cells, including platelets (Beachey *et al.*, 1977), erythrocytes (Beachey *et al.*, 1979a), lymphocytes (Beachey *et al.*, 1979b), PMN leukocytes (Courtney *et al.*, 1981), and epithelial cells (Beachey and Ofek, 1976). Erythrocytes bound by pneumococcal LTAs were found to be rendered susceptible to lysis both *in vitro* and *in vivo* when exposed to even their own complement system, pointing to the possibility of tissue damage triggered by LTA during bacterial infections (Hummell and Winkelstein, 1986). Other than binding to cells, LTAs from *S. mutans* strain BHT were found to exhibit high affinity for hydroxyapatite (Ciardi *et al.*, 1977), a feature of LTA that could enhance Gram-positive colonization on dental surfaces. Also, LTA extracts from *Enterococcus hirae* ATCC 9790 bind to calcified matrix, as well as to cells of neonatal rat parietal and long bones (O'Grady *et al.*, 1980). In a tissue culture study, LTA stimulated bone resorption (Hausmann *et al.*, 1975). Corroborating data come from rat experiments where severe inflammatory lesions and bone resorption were induced in the periodontal tissues of the rats after repeated intragingival injection of LTA from *S. mutans* (Bab *et al.*, 1979).

Apoptosis is essentially the programmed death of a cell without damage to adjacent cells. While this process occurs constantly in virtually all organs throughout life, it may also be involved in several diseases, including oral diseases and periradicular lesions (Satchell *et al.*, 2003). LTA from streptococci has been found to cause apoptotic cell damage in cell culture (Wang *et al.*, 2001) and in tissue culture studies (Schmidt *et al.*, 2001). The effects of various components and particularly of LTA of *E. faecalis* in causing apoptosis in relevant cell lines (osteoblasts, osteoclasts, periodontal ligament fibroblasts, macrophages, and neutrophils) merit investigation, since it may bring new insight into the nature of periradicular lesions involving *E. faecalis*.

Lipoteichoic acids isolated from strains of *E. faecalis* or from other Gram-positive bacteria have been reported to stimulate leukocytes to release several mediators which are known to play a role in various phases of the inflammatory response.

These include the release of TNF-α, interleukin 1 beta (IL-1β), interleukin 6 (IL-6) (Bhakdi *et al.*, 1991), and interleukin 8 (IL-8) (Saetre *et al.*, 2001) by cultured human monocytes and by human whole-blood leukocytes, respectively, the release of prostaglandin E2 (PGE2) by mouse peritoneal macrophages (Card *et al.*, 1994), the release of lysosomal enzymes by rat peritoneal macrophages (Harrop *et al.*, 1980), and the generation of superoxide anion by human monocytes (Levy *et al.*, 1990) (Fig.).

These factors have all been detected in periapical samples, and each has a well-known tissue-damaging (TNF-α, IL-1β, IL-6, PGE2, lysosomal enzyme, superoxide anion) or leukocyte-attracting (IL-8) property.

Change in vascular permeability is also an important phase in the course of inflammation, since extravasation of plasma, succeeded by diapedesis of circulating leukocytes, follows an increase in vascular permeability. LTA from *S. aureus* was shown to increase the vascular permeability in mice, probably through production of secondary mediators such as eicosanoids, platelet-activating factor, and histamine (Wada *et al.*, 2000). A recent study indicates that streptococcal LTA upregulates the expression of vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis, vascular permeability, and edema, in macrophages and pulp cells (Telles *et al.*, 2003). While an increase in vascular permeability is related to acute inflammation, angiogenesis is related more to chronic inflammation.

A proper autolytic activity appears to be necessary for the efficient killing of bacteria by cell-wall antibiotics. However, LTA has been found to inhibit the autolysis of isolated walls as well as intact cells of the former *Streptococcus faecalis*, now termed *Enterococcus hirae* ATCC 9790 (Cleveland *et al.*, 1976). Compared with the parent strain, autolytic-defective mutants of *E. hirae* ATCC 9790 showed increased survival after exposure to cell-wall antibiotics. Moreover, they exhibited decreased rates of autolysis when treated with detergents, suspended in lytic buffers, or when grown in medium depleted of essential nutrients in the presence of increased levels of cellular LTA and lipids (Shungu *et al.*, 1979). Thus, LTA appears to be associated with resistance against adverse conditions and may also be involved in resistance against root canal medicaments applied during endodontic treatment. Recently, LTA of *E. faecalis* was reported to be doubled in quantity during the viable but non-cultivable (VBNC) state, suggesting a role for LTA during this period (Signoretto *et al.*, 2000).

D-alanylation of the cell-wall-associated LTA could be important in bringing about phenotypical advantages for the bacteria. A mutant strain of *Streptococcus agalactiae*, deficient in the D-alanine moiety on the LTA, was more susceptible to killing by macrophages and neutrophils than the wild-type strain, and exhibited decreased virulence in animal models (Poyart *et al.*, 2003). Insertional inactivation of the gene *dltD* (responsible for expression of the protein that incorporates D-alanine into LTA) in *Lactobacillus casei* 102S resulted in enhanced antimicrobial activity of the disinfectants cetyltrimethylammonium bromide and chlorhexidine (Debabov *et al.*, 2000). The latter is also used as an endodontic disinfectant.

Finally, LTA has been considered as a constituent of the binding substance of *E. faecalis* that acts as the receptor on the recipient cell for aggregation substance produced by the donor cell. This presumption stems from experiments where free LTA isolated from *E. faecalis* inhibited pheromone-induced cell clumping by acting as a competitive inhibitor of the cellular-binding substance (Ehrenfeld *et al.*, 1986). Therefore, LTA can also be regarded as a molecule contributing to the virulence of *E. faecalis* through the facilitation of aggregate formation and plasmid transfer.

Extracellular Superoxide Production

Superoxide anion is a highly reactive oxygen radical involved in cell and tissue damage in a variety of disorders, including inflammatory diseases. Superoxide anion and other oxygen radicals exert a destructive effect on a wide variety of biological compounds such as lipids, proteins, and nucleic acids (Cross *et al.*, 1987). While production of superoxide by neutrophils and other phagocytic cells is essential for the killing of micro-organisms, it causes tissue damage at the site of inflammation. An altered balance between oxygen radical production by phagocytic cells in periapical lesions and its elimination was suggested to contribute to periapical damage and bone loss in chronic apical periodontitis (Marton *et al.*, 1993). Superoxide anion has also been shown to be produced by osteoclasts and involved in bone resorption (Key *et al.*, 1994). Furthermore, superoxide anion may react with a precursor in plasma to generate a factor that is chemotactic for neutrophils (Petrone *et al.*, 1980) (Fig.).

In addition to production by host cells, bacteria can also produce superoxide anion. Production of superoxide by a clinical isolate of a *Streptococcus* D sp. strain was lytic for erythrocytes (Falcioni *et al.*, 1981). Extracellular superoxide production has been reported to be a common trait in strains of *E. faecalis*. Among a total of 91 clinical and community isolates and type strains, 87 were found to produce detectable extracellular superoxide anion (Huycke *et al.*, 1996). Isolates associated with bacteremia or endocarditis produced significantly higher extracellular superoxide than those from the stool of healthy subjects (Huycke *et al.*, 1996). In a subcutaneous model, extracellular superoxide production was found to enhance the *in vivo* survival of *E. faecalis* in a mixed infection with *Bacteroides fragilis* (Huycke and Gilmore, 1997).

Gelatinase

Gelatinase is an extracellular zinc-containing metalloproteinase from *E. faecalis* which was first purified and described by Bleiweis and Zimmerman (1964). It can hydrolyze gelatine, collagen, fibrinogen, casein, hemoglobin, insulin, certain *E. faecalis* sex-pheromone-related peptides, and some other bioactive peptides (Mäkinen *et al.*, 1989).

Gelatinase, as a member of the matrix metalloproteinase (MMP) family, can also be produced by a wide variety of mammalian cells, including inflammatory cells, epithelial cells, fibroblasts, osteoclasts, etc. Acting on substrates similar to those of the bacterial gelatinase, host gelatinase plays a role in normal physiological processes, such as regulation of formation and remodeling of tissues through its extracellular matrix-degrading functions. However, unregulated MMP activity has been implicated in certain pathological states, such as invasion of cancer cells, arthritis, and periodontitis. Gelatinase (Gelatinase A, MMP-2; and Gelatinase B, MMP-9) levels were elevated in oral rinses, crevicular fluid, and whole saliva samples (Mäkelä *et al.*, 1994) and in gingival biopsy specimens (Soell *et al.*, 2002) from periodontitis patients compared with those in healthy subjects. Inhibition of gelatinase decreases the rate of bone resorption in tissue culture experiments (Hill *et al.*,

1994) and in experimental periodontal disease models (Ramamurthy *et al.*, 2002). Recently, host gelatinase was reported to be higher in inflamed pulps and periapical lesions than in healthy tissues (Shin *et al.*, 2002). Host gelatinase was also shown to have a significant effect in the degradation of dentin organic matrix (Tjäderhane *et al.*, 1998). Certain peptides, generated as a consequence of fragmentation of collagen, attract monocytes (Postlethwaite and Kang, 1976), macrophages, and neutrophils (Riley *et al.*, 1988; Laskin *et al.*, 1994) to the site of breakdown (Fig.). Furthermore, the collagen peptides were found to stimulate the release of destructive reactive oxygen species, hydrogen peroxide and the superoxide anion, and also the lytic enzymes, elastase and gelatinase, by macrophages (Laskin *et al.*, 1994) (Fig.). By analogy, collagen hydrolysis by the gelatinase of *E. faecalis* may therefore play an important role in the pathogenesis of periapical inflammation.

A zinc-containing metalloproteinase from *Legionella pneumophila* hydrolyzes similar substrates as the gelatinase of *E. faecalis*, and this enzyme has been associated with disease progression due to its cytotoxic and tissue-destructive potential and its inhibitory effects on phagocytes (Dowling *et al.*, 1992).

Another condition where *E. faecalis*-derived gelatinase can produce pathological alterations may be seen in the study by Gold *et al.* (1975), where the gelatin-liquefying strain 2SaR induced caries in rats, whereas this was not the case with non-proteolytic strains.

Animal studies indicate increased lethality of a gelatinase-producing *E. faecalis* strain compared with the isogenic strain deficient in gelatinase production (Singh *et al.*, 1998b).

In epidemiologic studies with human clinical isolates of *E. faecalis* (those isolated from hospitalized patients with infection at various sites), gelatinase production was detected in 45-68% of the isolates (Coque *et al.*, 1995; Elsner *et al.*, 2000; Kanemitsu *et al.*, 2001), and the gelatinase activity was higher in clinical isolates than in fecal isolates from healthy volunteers (Coque *et al.*, 1995).

Hyaluronidase

Hyaluronidase acts on hyaluronic acid (hyaluronate, hyaluronan) and is mainly a degradative enzyme that is associated with tissue damage as the consequence of its activity. It is found widely in nature, from mammalian cells such as spermatozoa to snake venom and to parasites such as leeches and hookworms. It is produced in high quantities by streptococci and other bacteria as well (Hynes and Walton, 2000). Hyaluronidase depolymerizes the mucopolysaccharide moiety of connective tissues, and so increases bacterial invasiveness. Strains of *Streptococcus pneumoniae* with low or no hyaluronidase were found to cause brain infections in mice only when they were inoculated together with exogenous hyaluronidase (Kostyukova *et al.*, 1995). Hyaluronidase was shown to be critical for the dissemination of *Treponema pallidum*, which is the causative micro-organism of syphilis (Fitzgerald and Repesh, 1987). Hyaluronidase activity is detected in culture supernatants of *Streptococcus intermedius* isolated from human pus, indicating its potential role in tissue degradation (Takao *et al.*, 1997).

Another role for hyaluronidase may be to supply nutrients for the bacteria, since the degradation products of its target substrates are disaccharides that can be transported and metabolized intracellularly by bacteria (Hynes and Walton, 2000). Hyaluronic acid as the substrate for hyaluronidase has also been detected in dentin (Jones and Leaver, 1974; Chardin *et al.*, 1990). Streptococci, isolated from carious dentin, can grow in medium containing only hyaluronic acid, suggesting that the bacteria may derive the essential carbon for their growth through hydrolysis of the substrate (Toto *et al.*, 1968). Production of hyaluronidase by streptococci and a strain of *E. faecalis* isolated from carious dentin could play a role in tissue destruction during the caries process (Parikh *et al.*, 1965). Bacteria isolated from infected root canals associated with apical periodontitis also produce hyaluronidase, and the hyaluronidase activity appears to be related to the degree (acute and subacute) of clinical symptoms (Hashioka *et al.*, 1994).

Hyaluronidase ('the spreading factor') is considered to facilitate the spread of bacteria as well as their toxins through host tissues. In addition to its own damaging effect, hyaluronidase may also pave the way for the deleterious effects of other bacterial toxins, thus increasing the magnitude of the damage. The presence of micro-organisms, including *E. faecalis*, in periapical lesions (Abou-Rass and Bogen, 1998; Sunde *et al.*, 2002) may also be related to the activity of a degrading bacterial enzyme such as hyaluronidase. It may act to facilitate the migration of bacteria from the root canal into the periapical lesion. Interestingly, a large number of species reported in the aforementioned studies are capable of producing hyaluronidase. However, due to the lack of studies concerning the role of hyaluronidase in enterococcal virulence, the contribution of this factor to the apical periodontitis caused by enterococci remains hypothetical.

Cytolysin

Formerly called hemolysin, cytolysin, as expressed by various isolates of *E. faecalis*, is most frequently a plasmid-encoded toxin, but it may also be chromosomally encoded (Ike and Clewell, 1992). Production and activation of cytolysin involves a series of elaborate stages. The lytic factor precursors $CylL_L$ (the long subunit) and $CylL_S$ (the short subunit) are ribosomally synthesized and modified post-translationally by CylM. The modified peptides are then proteolytically cleaved and secreted from the cell by CylB, an ABC transporter. The secreted peptide subunits $CylL_L'$ and $CylL_S'$ are further cleaved and activated extracellularly by CylA, a serine protease. Fully mature $CylL_L''$ and $CylL_S''$ are both required for the lysis of target cells (Haas and Gilmore, 1999). The cytolysin-producing bacterium itself is protected from lysis by the *cylI* gene product, through unknown mechanisms (Coburn *et al.*, 1999).

Among the target cells of cytolysin are the erythrocytes (Basinger and Jackson, 1968; Miyazaki *et al.*, 1993), PMNs and macrophages (Miyazaki *et al.*, 1993), and a broad range of Gram-positive, but not Gram-negative, organisms (Jackson, 1971; Jett and Gilmore, 1990). It has been hypothesized that if the bacteriocin effect of cytolysin of *E. faecalis* favors colonization of the Gram-negatives, there could be a shift to a bacterial flora usually associated with periodontal disease (Jett and Gilmore, 1990).

Recent studies investigated the influence of environmental factors on the expression of cytolysin genes. In one study, a quorum-sensing mechanism for production of cytolysin was identified (Haas *et al.*, 2002). According to this study, the products of two regulatory genes, *cylR1* and *cylR2*, work together to repress the transcription of the cytolysin structural genes. As soon as the level of one of the cytolysin subunits, $CylL_S''$ (the

fully mature form), reaches an extracellular threshold, derepression occurs and cytolysin expression is induced. Another study suggests that the genes $cylL_L$ and $cylL_S$, encoding the structural subunits of cytolysin, are regulated in response to changing oxygen conditions, and increased amounts of cytolysin are produced under anaerobic conditions (Day *et al.*, 2003). From an endodontic point of view, this finding is important, in that cells of *E. faecalis* may encounter anaerobic conditions in the root canal following depletion of oxygen by aerobes. Anaerobic conditions may also prevail in the layers of bacterial biofilms in the root canal, and *E. faecalis* has the capacity to produce biofilms (Distel *et al.*, 2002).

Epidemiological investigations partly support a role for cytolysin in disease occurrence. Ike *et al.* (1987) reported that approximately 60% of *E. faecalis* clinical isolates were hemolytic, in contrast to only 17% of *E. faecalis* isolates derived from fecal specimens from healthy individuals. In another study, *cylA* occurred more frequently among bacteremia isolates than in isolates from cases of endocarditis or from stools from healthy subjects (Huycke and Gilmore, 1995). In contrast, the study by Coque *et al.* (1995) did not reveal any difference in cytolysin incidence among *E. faecalis* isolates from endocarditis, bacteremia, or stool from healthy subjects. In another study, where only 16% of *E. faecalis* clinical isolates produced cytolysin, the role of this protein as a main virulence factor was concluded to be small (Elsner *et al.*, 2000). However, results from a recent study suggested that silent *cyl* genes from clinical isolates of *E. faecalis* may give a negative phenotypic profile (no hemolytic activity on blood agar plates), but environmental factors, such as those found in the infection site, may activate the genes (Eaton and Gasson, 2001).

Data from animal models (Ike *et al.*, 1984; Jett *et al.*, 1992, 1995; Chow *et al.*, 1993; Singh *et al.*, 1998b) and a nematode model (Garsin *et al.*, 2001) suggest cytolysin to be an important virulence factor. In a rabbit endophthalmitis experiment, antibiotic treatment against *E. faecalis* together with corticosteroid therapy was effective in cases of non-cytolytic strains in preventing visual loss as a consequence of tissue damage, whereas this therapy was useless in the case of infection with the cytolytic strain, suggesting a pathogenic role for cytolysin in endophthalmitis (Jett *et al.*, 1995).

AS-48

AS-48 is a plasmid-encoded peptide antibiotic originally isolated from *E. faecalis* S-48 (Martinez-Bueno *et al.*, 1990). AS-48 has been shown to exert lytic activity toward a broad range of Gram-positive and Gram-negative bacteria (Galvez *et al.*, 1989). The mode of action of AS-48 on target cells has been thought to be through molecular electroporation due to its high net-positive charge and through induction of ion permeation, which is accompanied by the collapse of the cytoplasmic membrane potential (Galvez *et al.*, 1991; Gonzalez *et al.*, 2000). Results of polymerase chain-reaction (PCR) investigations on independently isolated bacteriocin-producing *E. faecalis* strains indicated that bacteriocins produced by many *E. faecalis* strains were closely related or even identical to peptide AS-48 (Joosten *et al.*, 1997).

Other Bacteriocins

In addition to the bacteriocins 'cytolysin' and 'AS-48', Bc-48 (Lopez-Lara *et al.*, 1991), enterocin 226NWC (Villani *et al.*, 1993), enterocin 4 (Joosten *et al.*, 1996), enterococcin EFS2 (Maisnier-Patin *et al.*, 1996), bacteriocin 31 (Tomita *et al.*, 1996), bacteriocin 21 (Tomita *et al.*, 1997), enterocin EJ97 (Galvez *et al.*, 1998), enterocin 1071A and enterocin 1071B (Balla *et al.*, 2000), and enterocin SE-K4 (Eguchi *et al.*, 2001) have been isolated from various strains of *E. faecalis* and have been reported to have inhibitory action mainly on Gram-positive bacteria.

Conclusion

For a bacterium to be pathogenic, it must essentially be able to adhere to, grow on, and invade the host. It must then survive host defense mechanisms, compete with other bacteria, and produce pathological changes. With the virulence factors described above, *E. faecalis* appears to possess the requisites to establish an endodontic infection and maintain an inflammatory response potentially detrimental to the host. The virulence factors of *E. faecalis* and their functions are summarized in the Table, and a model of the endodontic disease related to the virulence factors is presented in the Fig.

Upon contamination of the root canal with the bacterium, it can colonize the dentinal walls, adhering to the mineral part, probably through LTA, and to the collagen through AS and other surface adhesins (Fig.). It may be that the most interesting among these surface adhesins is 'Ace', which is expressed by the bacterium under disease conditions and particularly under stress (Rich *et al.*, 1999). Bacteria face a variety of stressful conditions in the root canal, such as nutrient deficiency, toxins of other bacteria, and endodontic medicaments. These conditions may modulate the adhesin expression of the

TABLE
An Overview of the Virulence Factors of *E. faecalis* and Their Functions

Function	Factor	References
Adhesion and colonization	AS	Kreft *et al.*, 1992; Rodzinski *et al.*, 2001
	other surface adhesins	Rich *et al.*, 1999; Shankar *et al.*, 2001
	LTA	Ciardi *et al.*, 1977
Resistance to host defense	AS	Rakita *et al.*, 1999; Süßmuth *et al.*, 2000
Inhibition on other bacteria	cytolysin	Jett and Gilmore, 1990
	AS-48	Galvez *et al.*, 1989
	other bacteriocins	References in the text
Tissue damage	LTA	Hausmann *et al.*, 1975; Bab *et al.*, 1979
	extracellular superoxide anion	Key *et al.*, 1994
	gelatinase	Mäkinen *et al.*, 1989; Hill *et al.*, 1994
	hyaluronidase	Takao *et al.*, 1997
	cytolysin	Jett *et al.*, 1992
Induction of inflammation	sex pheromones	Sannomiya *et al.*, 1990; Ember and Hugli, 1989
	LTA	Bhakdi *et al.*, 1991; Card *et al.*, 1994

bacterium. In addition, leakage of serum into the root canal can induce the expression of AS and other carbohydrate moieties, thereby increasing the adhesiveness of the bacterium. Adhesion to dentin and penetration along dentinal tubules by *E. faecalis* may serve as a means of protection from endodontic medicaments. An example is calcium hydroxide. When calcium hydroxide is placed in the root canal, the pH decreases sharply toward deeper dentinal zones (Tronstad *et al.*, 1981; Nerwich *et al.*, 1993). Thus, bacteria that have penetrated more deeply into the dentinal tubules and established footholds peripheral to the main root canal are at an advantage. Another mechanism by which *E. faecalis* survives may be through LTA, which has been associated with resistance of the bacterium against a variety of lethal conditions (Shungu *et al.*, 1979).

Since *E. faecalis* suppresses the growth of other bacteria with its cytolysin, AS-48, and other bacteriocins, the activity of these toxins against Gram-positive and -negative bacteria can explain, in part, the low number of other species in persistent endodontic infections where *E. faecalis* is dominant. The latter factors are not believed to be pathogenic in humans. However, along with cytolysin, they facilitate the dominance of *E. faecalis* in a mixed infection and serve as means to obtain ecological advantages which can result in disease in man.

The root canal is hardly a nutrient-rich medium, but *E. faecalis* may derive the energy it needs from the hyaluronan present in the dentin through degradation by hyaluronidase. *E. faecalis* may also feed on serum components present in the fluid in the dentinal tubules. Moreover, an inadequate apical seal of root fillings may allow serum to flow into the root canal. Therefore, it seems that, even in a well-debrided and coronally well-sealed root canal, remaining or arriving cells of *E. faecalis* may still grow and utilize local sources of energy and nutrients.

Production of extracellular superoxide and release of the lytic enzymes gelatinase and hyaluronidase and the toxin cytolysin by *E. faecalis* can cause direct damage in the dentinal as well as in the periapical tissues (Fig.). In contrast, *E. faecalis* can also induce host-mediated tissue damage in the periradicular tissues. Since cells of *E. faecalis* in the dentinal tubules cannot be reached and eliminated by the cells of the host defense system, they may elicit a permanent provocative effect on these cells. PMN leukocytes, lymphocytes, monocytes, and macrophages are stimulated by a group of virulence factors of *E. faecalis*, which will contribute to the periradicular damage.

It has been proposed that, since strains of *E. faecalis* frequently harbor plasmids determining antibiotic resistance, cytolysin, and/or bacteriocin, they may represent a reservoir of genetic information available to other bacteria in the intestine (Clewell and Weaver, 1989). This applies to the root canal microbiota as well. While antibiotic resistance and other virulence traits can be disseminated by means of the sex-pheromone-responsive plasmid transfer among the strains of *E. faecalis*, gene transfer is also possible from *E. faecalis* to bacteria of other species or even of other genera through sex-pheromone-independent conjugation. So far, there is no information on whether multiple strains of *E. faecalis* simultaneously participate in endodontic infections in utilizing the sex-pheromone-related gene transfer. However, *E. faecalis* frequently colonizes the root canal together with bacteria of other species and/or genera, and it may use the latter pathway of gene transfer. This is associated mainly with the transfer of antibiotic resistance genes. Thus, bacteria resistant to multiple antibiotics can be generated within the root canals, where *E. faecalis* plays a pivotal role. It has been reported that micro-organisms from the root canal can be seeded into the bloodstream during endodontic treatment, and this has the potential to bring about serious systemic diseases such as endocarditis, brain abscesses, and septicemia, particularly for compromised patients (Debelian *et al.*, 1994, 1995). Although this may be a rare clinical occurrence, there are reported cases related to endodontic infections and endodontic treatment (Henig *et al.*, 1978; Lee, 1984; Green and Haisch, 1988). In this context, bacteria resistant to multiple antibiotics pose particular problems. Indeed, in marginal periodontitis refractory to conventional treatment, an increased prevalence of bacteria resistant to antibiotics may be found (Handal *et al.*, 2003).

It cannot be excluded that bacteria may pass through the apical foramen to the periradicular lesion during the course of endodontic infection and elicit host responses. However, the focus of infection is the root canal and the dentinal tubules, which are inaccessible to the elements of the host defense system. Therefore, treatment or preventive procedures should mainly include local, rather than systemic, means. In addition to disinfectants, physical removal of cells of *E. faecalis* through debridement of the root canal remains essential, since remnants containing LTA may still sustain the inflammation.

The use of agents blocking the expression of virulence genes or modulating their products may find a role in future treatments of persistent endodontic infections with *E. faecalis*. For example, sensitization of the bacteria to root canal medicaments, which are otherwise ineffective, particularly through targeting the LTA synthesis or d-alanylation of the LTA chain, may be possible, but a better understanding of the regulation of the virulence genes is necessary.

Another possible preventive measure to avoid invasion of the dentinal tubules by *E. faecalis* may be through disruption of the dentinal collagen, the target for the adhesins. Enzymatic modulation is one possible way of altering the collagen. Similarly, proteinaceous bacterial adhesins may be targeted by protein-digesting agents such as trypsin. These methods have been tested *in vitro* and resulted in decreased adherence of *E. faecalis* to collagen-coated surfaces (Xiao *et al.*, 1998).

In conclusion, this review has dealt with the virulence factors of *E. faecalis* that may enable the bacterium to establish an endodontic infection and maintain a periradicular inflammation. A model of endodontic disease related to these factors has been proposed. The pathogenesis of the periradicular lesions is definitely a very complex process that may involve a large number of host and microbial factors (Torabinejad *et al.*, 1985; Stashenko, 1998; Takahashi, 1998). In the present context, only those aspects of the immune and inflammatory events likely to occur within the periradicular lesion in relation to the virulence factors of *E. faecalis* have been discussed. It has been established that the primary periradicular lesion is a consequence of a mixed microbial flora rather than solely of *E. faecalis*. However, in apical periodontitis that persists despite root canal treatment, *E. faecalis* is frequently the dominant, sometimes the only, pathogen, suggesting that this species alone has the potential to maintain root canal infection and periradicular lesion. A better understanding of the role of the virulence factors of *E. faecalis* in endodontic infections may help in the development of new strategies to prevent or to eliminate the infection by this species, thereby improving treatment results in endodontics.

Acknowledgment

The financial support of The Research Council of Norway is gratefully acknowledged.

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