CHAPTER 1

INTRODUCTION

1.1 Rationales

The main goals of endodontic are the prevention and treatment of diseases of the dental pulp and periradicular tissues. The inflammatory reaction is first initiated by bacterial antigens interacting with the local immune system. The endodontic environment provides a selective habitat for the establishment of mixed, predominantly anaerobic, flora (Nair, 2004). This habitat-adapted polymicrobial community residing in the root canal has several biological and pathogenic properties, such as antigenicity, mitogenic activity, chemotaxis, enzymatic histolysis, and activation of host cells (Stashenko, 1990). The microbial invaders in the root canal can advance, or their products can egress, into the periapex. The microbial factors and host defense forces encounter, clash with, and destroy much of the periradicular tissues (Stashenko, 1990), resulting in the formation of various categories of apical periodontitis lesions (Nair, 2004).

Apical periodontitis is an inflammatory process in the periradicular tissues caused by microorganisms in the necrotic root canal (Kakehashi et al., 1965). The treatment of apical periodontitis consists of eliminating infection from the root canal and preventing re-infection by a hydraulic seal of the root canal space. When the treatment is done properly, healing of the periradicular lesions usually occurs with osseous regeneration, which is characterized by gradual reduction and resolution of

the radiolucency on subsequent follow-up radiographs (Sjogren et al., 1990; Sundqvist et al., 1998).

Nevertheless, endodontic treatment can fail for several reasons. It is generally acknowledged that most failures occur when treatment procedures have not reached a satisfactory standard for the control and elimination of infection (Sjogren et al., 1997; Katebzadeh et al., 2000). Common problems that may lead to endodontic failure include inadequate aseptic control, poor access cavity design, missed canals, inadequate instrumentation, and leaking of temporary or permanent fillings (Sundqvist et al., 1998).

Enterococcus faecalis (*E. faecalis*), a Gram-positive bacterium, has been frequently recovered from root-filled teeth with refractory apical periodontitis. It is the most frequently isolated species and usually also the predominant species in the canal (Sundqvist et al., 1998; Pinheiro et al., 2003; Rocas et al., 2004; Gomes et al., 2006). The high frequencies of isolation of *E. faecalis* have been reported at 67%-76% by PCR detection (Rocas et al., 2004; Sirqueira and Rocas, 2004; Gomes et al., 2008). Many of these teeth are asymptomatic and remain undiscovered for years until a routine radiograph may reveal a periradicular lesion of chronic apical periodontitis (Peciuliene et al., 2000). Patients may have experienced recurrent mild pain from the affected tooth, whereas in other cases a severe flare-up may occur years after root canal treatment.

E. faecalis is well adapted for survival and persistence in a variety of adverse environments. *E. faecalis* may feed on serum components present in the fluid in the dentinal tubules (Figdor et al., 2003). Moreover, an inadequate apical seal between a root filling and a canal wall may allow serum to flow into the root canal. In an *in*

vitro study, *E. faecalis* has been shown to invade dentinal tubules (Love et al., 1997). It can colonize root canal and survive without the support of other bacteria (Fabricius et al., 1982; Sobrinho et al., 1998). It is resistant to the antimicrobial effects of calcium hydroxide, probably partly due to an effective proton pump mechanism which maintains optimal cytoplasmic pH levels (Tanriverdi et al., 1997).

Lipoteichoic acids (LTA), isolated from strains of *E. faecalis* or from other Gram-positive bacteria (Wicken and Knox, 1975), 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 tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) by cultured human monocytes (Bhakdi et al., 1991), and the release of interleukin-8 (IL-8) by human whole-blood leukocytes (Saetre et al., 2001). *E. faecalis* can induce hydrolytic enzymes from the polymorphonuclear (PMN) cells (Reynaud af Geijersstam et al., 2005).

Bacterial products induce the local cells to secrete high levels of several cytokines and proteases that degrade extracellular matrix (ECM), resulting in tissue destruction (Kinane and Lappin, 2001; Seymour and Gemmell, 2001). Among host proteases that target the ECM, matrix metalloproteinases (MMPs), a group of enzymes produced by connective tissue cells, such as the fibroblast, are capable of degrading collagen (types I, II, III, and IV), proteoglycans, and elastin (Birkedal-Hansen et al., 1993). Under normal physiological conditions, the activities of MMPs are precisely regulated at the levels of transcription, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors (Nagase and Woessner, 1999; Sternlicht and Werb, 2001). A loss of

activity control may result in diseases, such as arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis.

The activation of MMPs is regulated by a group of endogenous proteins, named tissue inhibitors of metalloproteinases (TIMPs) that are each capable of inhibiting almost every member of the MMP family in a non-specific way (Baker et al., 2002). Usually, the TIMPs are in balance with the MMPs and the matrix is remodeled in a highly regulated fashion. However, in many disease processes the levels of MMPs are elevated without a concomitant increase in TIMPs, resulting in tissue destruction (Nawrocki et al., 1997). It is thought that MMPs and TIMPs are involved in the physiological turnover of periodontal tissues, and MMPs appear to be involved in tissue destruction in periodontium (Golub et al., 2001).

Collagenases degrade interstitial collagen to produce denatured interstitial collagen, which is degraded by gelatinase, a type IV collagenase. Gelatinase includes MMP-2 and MMP-9. MMP-2 is mainly secreted by fibroblasts, whereas MMP-9 is mainly secreted by polymorphonuclear leukocytes and keratinocytes. Many reports have demonstrated the presence of MMP-2 and MMP-9 in gingival biopsy specimens and in gingival crevicular fluid in patients with periodontitis (Birkedal-Hansen, 1993; Tervahartiala et al., 2000; Kiili et al., 2002). Increased levels of MMP-2 and MMP-9 have been observed in gingival crevicular fluid from teeth with periradicular lesions (Belmar et al., 2008). Pro-MMP-2 has been expressed in clinically normal and diseased tissues, whereas active-MMP-2 has been detected only in tissues obtained from patients with clinical disease (Korostoff et al., 2000).

Apical periodontitis is the consequence of a dynamic encounter between root canal microbials and host defense (Nair, 1997). Periodontal ligament (PDL)

fibroblasts are candidate host cells, which are activated by bacterial antigens, and play an important role in the PDL degradation process. PDL fibroblasts function as accessory immune cells and are capable of synthesizing cytokines and enzymes that are associated with tissue destruction in response to bacterial products or inflammatory cytokines (Quintero et al., 1995; Agarwal et al., 1998). Therefore, these findings strongly suggest the involvement of PDL fibroblasts in periradicular tissue destruction.

Up to now, almost all of the *in vitro* and *in vivo* studies have substantiated the relationship of MMP-2 to periodontal disease. Few studies have focused on MMP-2 expression of Gram-negative bacterial products-treated the periodontal ligament fibroblasts (Choi et al., 2001; Chang et al., 2002; Pattamapun et al., 2003; Tiranathanagul et al., 2004; Zhou and Winsor, 2006), while the relation of MMP-2 in the periodontal ligament fibroblasts in response to bacterial products from Gram-positive bacteria have been scarcely investigated.

1.2 Objective

This study was designed to determine the effects of *E. faecalis*, a Gram-positive bacterium, and the dominant species, detected in endodontically failed teeth, on MMP-2 expression and activation in cultured fibroblasts isolated from human periodontal ligament.

1.3 Scope of study

The study was performed with an *in vitro* model of cultured fibroblasts from human periodontal ligament. To verify the hypothesis that bacterial products of

E. faecalis can up-regulate MMP-2 expression and activation, three methods were employed. Gelatin zymography was used to analyze MMP-2 activation. The reverse transcription polymerase chain reaction (RT-PCR) and the western blot analysis were used to analyze specific gene expression (MMP-2, MT1-MMP and TIMP-2) at transcriptional and translational levels, respectively.

1.4 Literature review

1.4.1 Periodontal ligament

The periodontal ligament tissue consists of cells and an extracellular component comprising collagenous and non- collagenous matrix constituents (McKee et al., 1996). The cells include fibroblasts, epithelial cell rests of Malassez, monocytes and macrophages, undifferentiated mesenchymal cells and cementoblasts. The extracellular component consists mainly of well-defined collagen fiber bundles embedded in an amorphous background material, known as ground substance. The principal cells of the periodontal ligament are fibroblasts (Nanci and Bosshardt, 2006). Although all fibroblasts look alike microscopically, heterogeneous cell populations exist between different connective tissues and also within the same connective tissue. The fibroblasts of the periodontal ligament are characterized by their rapid turnover of the extracellular component, in particular, collagen (Lekic and McCulloch, 1996).

Periodontal ligament fibroblasts are large cells with an extensive cytoplasm containing an abundance of associated organelles with protein synthesis and secretion. They have a well-developed cytoskeleton and show frequent adherens and gap junctions, reflecting the functional demands placed on the cells. Ligament

fibroblasts are aligned along the general direction of the fiber bundles and extend cytoplasmic processes that wrap around the bundles. The collagen fibrils of the bundles are continuously being remodeled by fibroblasts, which are capable of simultaneously synthesizing and degrading collagen. PDL fibroblasts function as accessory immune cells and are capable of synthesizing cytokines and enzymes that are associated with tissue destruction in response to bacterial products or inflammatory cytokines (Quintero et al., 1995; Agarwal et al., 1998).

The predominant collagens of the periodontal ligament are types I, III, and XII, with individual fibrils having a relatively smaller average diameter than do tendon collagen fibrils (Nanci and Bosshardt, 2006). The size difference is believed to reflect the relatively short half-life of ligament collagen, resulting in there being less time available for fibrillar assembly. The vast majority of collagen fibrils in the periodontal ligament are arranged in definite and distinct fiber bundles, and these are termed principal fibers. Each bundle resembles a spliced rope. Individual strands can be continually remodeled while the overall fiber maintains its architecture and function (Berkovitz, 1990).

Collagen destruction in periodontal diseases is mediated by metalloproteinases such as collagenase and stromelysin released by cells of the periodontium. Such proteolytic destruction can be induced by interleukin-1, whose production may not be dependent on a specific microbial flora but may be triggered by a number of organisms (Meikle et al., 1986).

1.4.2 Matrix metalloproteinases (MMPs)

MMPs are members of a group of enzymes that can break down proteins, such as collagen, that are normally found in the spaces between cells in

tissues (i.e., extracellular matrix proteins). Because these enzymes need zinc and calcium atoms to work properly, they are called metalloproteinases. MMPs from vertebrate species are given numbers (i.e. MMP-1, MMP-2, etc.), and MMPs from invertebrates rely on trivial names.

The MMP family consists of at least 26 members (Nagase and Woessner, 1999), all of which share a common catalytic core with a zinc molecule in the active site (Hirose et al., 1993). They are often grouped according to their modular domain structure (Figure 1.1). MMPs are produced as zymogens, with a signal sequence and propeptide segment that must be removed during activation (Harper et al., 1971). The propeptide domain contains a conserved cysteine, which chelates the active zinc site. An exception is MMP-23 which lacks this conserved cysteine, and has a very different propeptide domain (Park et al., 2000). A subset of MMPs, including the membrane type MMPs (MT-MMPs), as well as MMP-11, MMP-21, MMP-23, and MMP-28, is thought to be cleaved by the PACE/Furin family of enzymes. Two of the MMPs (MMP-2 and MMP-9) contain three units of fibronectin type-II like domain inserted into the catalytic domain, presumably to enhance substrate binding (Shipley et al., 1996).

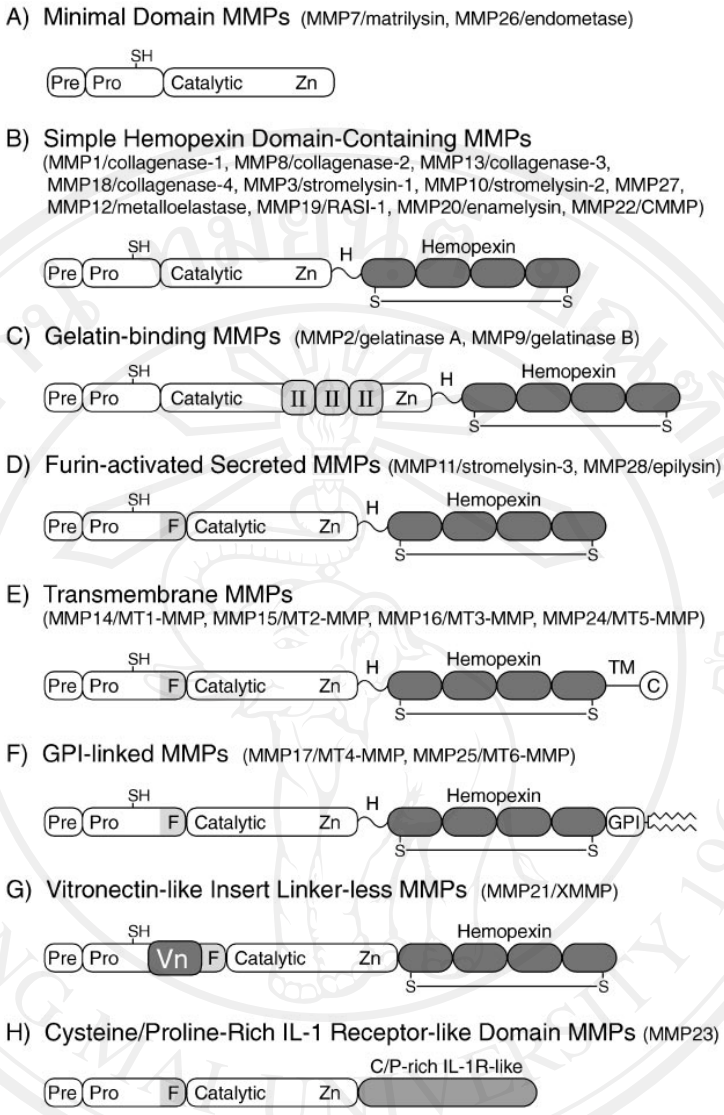

Figure 1.1 Structural domains of matrix metalloproteinases (Sterlicht and Werb, 2001)

The catalytic domain dictates cleavage site specificity through its active site cleft, through specificity sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site itself (Overall, 2001). All but two MMPs, MMP-7 and MMP-26, contain a regulatory subunit, the hemopexin domain,

separated from the catalytic domain by a variable hinge region (Park et al., 2000).

The hemopexin domain is thought to confer much of the substrate specificity to the MMPs, and is involved in activation as well as inhibition of the MMPs. The hinge region also confers specificity to the MMPs, either by direct binding or by setting the orientation of the hemopexin domain and the catalytic domain (Knauper et al., 1997). Final activation of the MMPs often includes shedding of the hemopexin domain, and the isolated hemopexin domain has been shown to inhibit intact MMPs. This hemopexin domain feedback might lead to direct down-regulation of MMPs activity, or may keep MMPs from associating with ECM or substrate.

The membrane-type matrix metalloproteinases (MT-MMPs) are localized to the cell surface (Sato and Seiki, 1996). Four of the MT-MMPs contain hydrophobic transmembrane domains (MMP-14, MMP-15, MMP-16, MMP-24), followed by a cytoplasmic domain. The other two MT-MMPs (MMP-17, MMP-25) lack cytoplasmic domains and are thought to be glycosylphosphatidylinositol (GPI) - anchored to the cell surface (Kojima et al., 2000). The cytoplasmic domain is thought to be involved in cytoskeletal signaling cascades, and may be directly phosphorylated by various kinase cascades. MMP-23 is believed to contain a unique transmembrane domain in the propeptide region, which may allow for a distinctly different attachment and activation cascade than the other MMPs.

MMPs are controlled by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Bauer et al., 1975). There are currently four known TIMPs, and they operate with different inhibition efficiencies against the different MMPs. The four TIMPs are also differentially expressed in tissues. TIMP-1 is

localized in the bone and plays a more specific role in bone development. TIMP-2 is constitutively produced, as is MMP-2, with which it is normally paired. This pairing is in fact required for the on-demand activation of MMP-2 (Brassart et al., 1998). The TIMPs are slow, tight-binding inhibitors with low nanomolar inhibition constants. TIMP-3 is localized in the ECM, and TIMP-4 is localized mostly in vascular tissue. TIMPs may be responsible for inhibiting a large family of proteinases *in vivo* (Ramnath and Creaven, 2004).

Substrate specificity for the MMPs is not yet fully characterized. Known substrates include most of the ECM components (fibronectin, vitronectin, laminin, entactin, tenascin, aggrecan, myelin basic protein, etc). The collagens (types I-X and XIV) have all been shown to be substrates of different MMPs, with greatly different efficacies (Overall, 2001). In addition to connective tissue and ECM components, proteinase inhibitors such as α_1-proteinase inhibitor, antithrombin-III and α_2-macroglobulin are selectively cleaved by MMPs. Intact type I collagen has also been used as a substrate for some MMPs. MMP-1 cleaves intact triple helical collagen efficiently, but does not work well on other substrates. Other MMPs, such as MMP-3 and MMP-7, cleave a broad range of substrates.

MMPs are involved in wound healing, angiogenesis, and tumor cell metastasis (Nelson et al., 2000; Sternlicht et al., 2000). The expression and activity of MMPs in adult tissues is normally quite low, but increases significantly in various pathological conditions that may lead to unwanted tissue destruction, such as inflammatory diseases, tumor growth and metastasis (Sorsa et al., 2004). MMPs have a marked role also in destructive oral diseases. MMP-1, -2, -3, -8, and -9 have been found in human inflammatory periodontal biopsy specimens (Birkedal-Hansen, 1993;

Reynolds, 1996; Westerlund et al., 1996). MMP-2 has been considered a major enzyme involved in the turnover of ECM (Creemers et al., 1998).

1.4.3 Matrix metalloproteinase-2 (MMP-2)

MMP-2 (type IV collagenase; gelatinase A; 72 kDa gelatinase) degrades denatured collagens, gelatin, as well as type IV collagen, a principal structural component of basement membrane (Aimes and Quigley, 1995). MMP-2 can digest type I, II and III collagens (Patterson et al., 2001). Most cells of mesenchymal origin have the ability to synthesize and secrete MMP-2 in pro-form (pro-enzyme), and require activation for catalytic activity, possibly by cleavage of the N-terminal prodomain. MMP-2 is secreted mainly by fibroblasts, predominant cells in connective tissues. Using *in situ* hybridization and immunohistochemistry, it was demonstrated MMP-2 is expressed in healthy gingiva (Tervahartiala et al., 2000). It has been reported that healthy gingiva contains only pro-MMP-2 (Korostoff et al., 2000).

MMP-2 was constitutively expressed by many cell types (Brown et al., 1996). MMP-2 function is controlled at three levels; transcription, activation and inhibition. MMP-2 activation is different from that of other MMPs, since MMP-2 activation requires the formation of a trimolecular complex consisting of MT1-MMP, tissue inhibitor of metalloproteinase-2 (TIMP-2) and MMP-2 on the cell surface (Strongin et al., 1995). Some studies have shown that the plasma membrane fraction from cultured cells could activate pro-MMP-2 (Azzam and Thompson, 1992).

Structurally, the catalytic core of MMP-2 is a polypeptide, consisting of a catalytic domain, which includes three repeats of fibronectin type II domains. The fibronectin domain functions as a binding site for the substrate, consisting of

collagens and laminin (Allan et al., 1995). Among all MMPs, MMP-2 has a unique activation mechanism. Activation of pro-MMP-2 has been shown to occur at the cell surface. The N-terminal domain of TIMP-2 binds to MT1-MMP prior to using the C-terminal domain binding to the hemopexin domain of pro-MMP-2, forming a three-molecular complex. Then a nearby MT1-MMP that is free of TIMP-2 cleaves the pro-domain of pro-MMP-2, at the Asn37-Leu38, followed by an autocatalytic conversion of the intermediate into a fully active enzyme (Murphy et al., 1999; Atkinson et al., 2001).

Pro-MT1-MMP is activated during transport to the cell surface by an intracellular furin-like serine proteinase, at the cell surface by plasmin, or by non-proteolytic conformational changes. The activated MT1-MMP is then inhibited by TIMP-2 and the hemopexin domain of pro-MMP-2 binds to the C terminal portion of TIMP-2 to form a trimolecular complex. An uninhibited MT1-MMP then partially activates the pro-MMP-2 by removing most of the MMP-2 propeptide. The remaining portion of the propeptide is removed by a separate MMP-2 molecule at the cell surface to yield fully active mature MMP-2 (Deryugina et al., 2001) (Figure 1.2). Mature MMP-2 can then be released from the cell surface or bound by another cell surface MMP-2-docking protein. It can also be inhibited by another TIMP-2 molecule or left in an uninhibited active state depending on local molar ratios of MMP-2 to TIMP-2 (Overall et al., 2000)

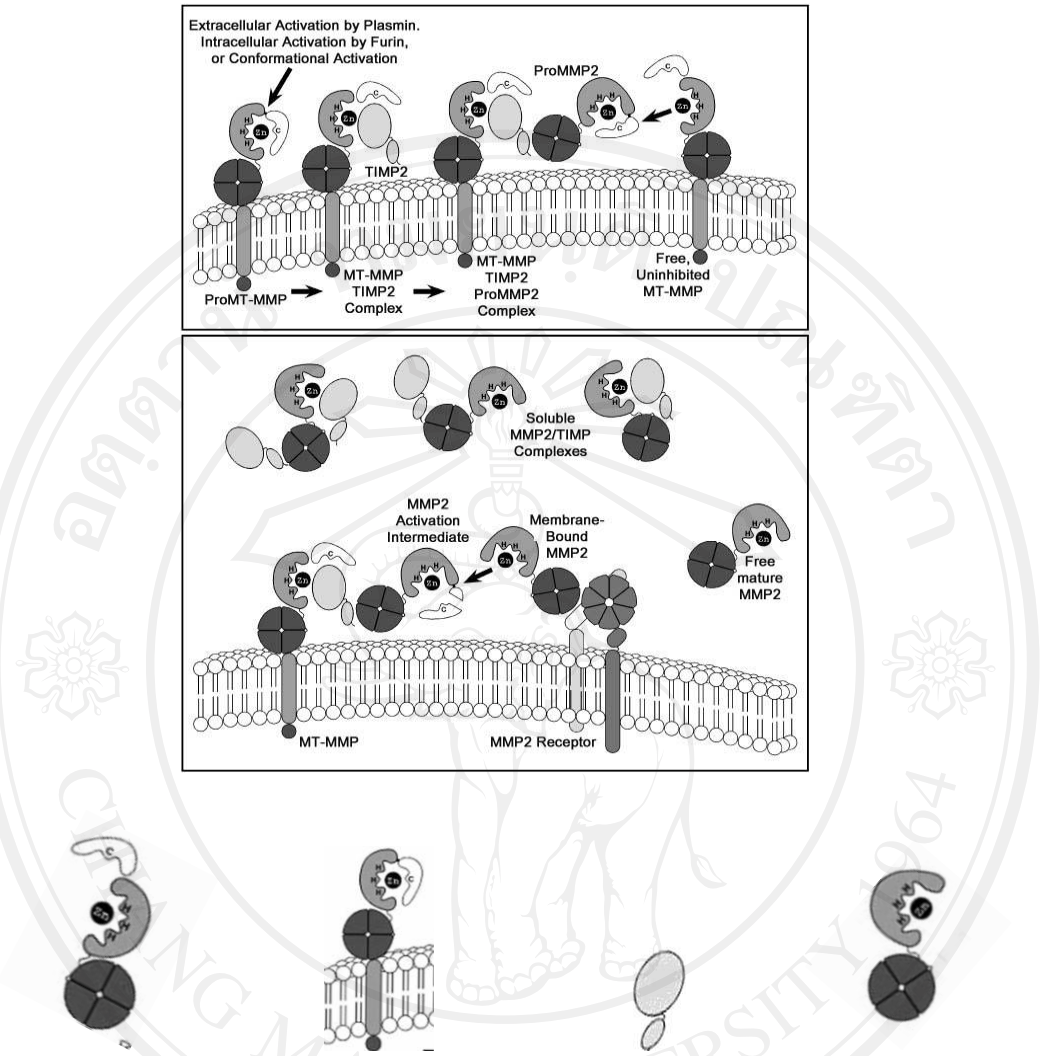

Figure 1.2 Cell surface activation of MMP-2 (Sternlicht and Werb, 2001)

TIMP-2 is known to be a bifunctional molecule, it stimulates MMP-2 activation at low concentration, whereas it inhibits activation at high concentration (Hernandez-Barrantes et al., 2000). Pericellular TIMP-2 concentration is critical for MT1-MMP mediated activation, such that insufficient TIMP-2 results in failure to localize pro-MMP-2 to the cell surface, whereas excess inhibitor reduces the population of free MT1-MMP needed to activate pro-MMP-2 (Morrison et al., 2001).

Obviously, from the MMP-2 activation model, the balance of these molecules: MMP-2, TIMP-2 and MT1-MMP, is important to stipulate the level of MMP-2 activation and ECM-degradation ability of MMP-2. The imbalance of these molecules, especially MMP-2 and TIMP-2, is possibly involved in the invasiveness of cancer, such as malignant glioma (Hur et al., 2000). Some studies have demonstrated the existence of an alternative TIMP-2-independent pathway for MMP-2 activation involving MT2-MMP (Morrison et al., 2001).

1.4.4 *Enterococcus faecalis* (*E. faecalis*)

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* are aggregation substance (AS), surface adhesions, sex pheromones, lipoteichoic acid (LTA), extracellular superoxide, gelatinase, hyaluronidase, and cytolysin (Kayaoglu and Orstavik, 2004). 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 (Joosten et al., 1997).

The most interesting among these surface adhesins is 'Ace', which is expressed by the bacterium under diseased 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 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 (Kreft et al., 1992). Adhesion to dentin and penetration along dentinal tubules by *E. faecalis*

may serve as a means of protection from endodontic medicaments (Haapasalo and Orstavik, 1987; Love and Jenkinson, 2002).

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 Gram-negative bacteria can explain, in part, the low number of other species in persistent endodontic infections where *E. faecalis* is dominant.

However, along with cytolysin, they facilitate the dominance of *E. faecalis* in a mixed infection and serve as a 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 (Hynes and Walton, 2000). *E. faecalis* may also feed on serum components present in the fluid in the dentinal tubules (Figdor et al., 2003). Moreover, an inadequate apical seal between a root filling and a canal wall 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.

PMN leukocytes, lymphocytes, monocytes, and macrophages are stimulated by a group of virulence factors of *E. faecalis*, which contribute to the periradicular damage (Reynaud af Geijersstam et al., 2005). 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 (Costa et al., 2003). 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.

Gelatinase from *E. faecalis* was first purified by Bleiweis and Zimmerman (Bleiweis and Zimmerman, 1964). The gelatinase of *E. faecalis* OG1-10 has recently been isolated and characterized (Makinen et al., 1989). It is an extracellular Zn+ endopeptidase (metalloendopeptidase II; microbial proteinase, EC 3.4.24.4) with a molecular weight of approximately 33,030 Dalton (Makinen et al., 1989), and 34,582 Dalton (Su et al., 1991).

E. faecalis has been reported in 67-76% of root-filled teeth with periradicular lesions by polymerase chain reaction (PCR) (Sundqvist et al., 1998; Pinheiro et al., 2003; Rocas et al., 2004; Gomes et al., 2006). Many of these teeth are asymptomatic and remain undiscovered for years until a routine radiograph may reveal a periradicular lesion of chronic apical periodontitis (Peciuliene et al., 2000).