

**GENOTYPIC AND PHENOTYPIC
CHARACTERIZATION OF *PORPHYROMONAS*
GINGIVALIS IN RELATION TO VIRULENCE**

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To the memory of my dear mother

To my father and sisters

With love to my wife, Tsuyumi and our newborn daughter, Yuna

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Genotypic and phenotypic characterization of *Porphyromonas gingivalis* in relation to virulence

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ABSTRACT

The present thesis was designed to increase the knowledge on the virulence potential of *Porphyromonas gingivalis* as a putative periodontal pathogen. *P. gingivalis* was selected to be the model species for a periodontal pathogen based on its characteristic of expressing a number of significant and unique virulence factors and on the considerable genetic heterogeneity of this species. The hypothesis of the present studies was that the pathogenic potential of *P. gingivalis* differs among this species and that certain clonal types of *P. gingivalis* have a more pathogenic capacity than others. The over all aim of this thesis was to investigate the phenotypic and genotypic characteristics and virulence properties of the species *Porphyromonas gingivalis*.

Material and Methods:

- Phenotypic heterogeneity of *P. gingivalis* species was evaluated by colony morphology, biochemical tests, enzymatic profiles, gas-liquid chromatography, antibiotic susceptibility, SDS-PAGE profiling of cell wall proteins and serotyping by monoclonal antibodies (Paper I).
- The diversity of whole chromosomal DNA among *P. gingivalis* species was evaluated by using amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) genotyping assays (Paper II).
- The variations of specific virulence biotypes based on *fimA*, *rgpA* and *kgp* genes and capsular K-antigens in *P. gingivalis* species were evaluated (Paper III).
- The interaction of *P. gingivalis* species with epithelial was evaluated by KB epithelial cell binding assay (Paper IV).

Results:

- *P. gingivalis* strains showed a strong homogeneity in relation to biochemical tests and antibiotic susceptibility. Furthermore, the majority of *P. gingivalis* strains displayed monoclonal antibodies (MAbs) serotype A, while serotype B was uncommon (Paper I).
- *P. gingivalis* isolated from Swedish subjects with periodontitis and periodontal abscess exhibited a wide variety of genotypes with weak clustering pattern. No predominant genotype at the whole chromosomal DNA level was present among these *P. gingivalis* (Paper II).
- Chronic periodontitis is not associated with a particularly virulent genotype of *P. gingivalis*. A highly virulent genotype (e.g. strain W83) of *P. gingivalis* can be detected in certain periodontitis subjects (Paper III).
- All strains showed binding capacity to host epithelial cells. Encapsulated *P. gingivalis* compared to non-encapsulated strains displayed a significantly lower binding capacity to host cells. No significant difference in binding and invasion was found between specific virulent genotypes. Thus, the two major virulence groups within *P. gingivalis* were mainly related to the presence/absence of a capsule structure of this organism (Paper IV).

In conclusion:

P. gingivalis isolates from Swedish periodontal disease cases express a considerable homogeneity in most phenotypic characteristics, although variations were found in colony morphology and MAbs and capsular antigen types. On the genotype level a considerable heterogeneity was found both at whole chromosomal level as for specific virulence genes. The studies support that there is generally a non-clonal structure of *P. gingivalis* although some specific virulent clones might be found infrequently in periodontitis. A capsule seems to be of particular importance for *P. gingivalis* pathogenicity.

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

- I. Dahlén G, Gmür R, Yoshino T. Phenotypes, serotypes and antibiotic susceptibility of Swedish *Porphyromonas gingivalis* isolates from periodontitis and periodontal abscesses. *Oral Microbiol Immunol* 2007;21 (in press).
- II. Yoshino T, Laine ML, van Winkelhoff AJ, Dahlén G. Genotypic characterization of *Porphyromonas gingivalis* isolated from Swedish patients with periodontitis and periodontal abscesses. *Oral Microbiol Immunol* 2007;21 (in press).
- III. Yoshino T, Laine ML, van Winkelhoff AJ, Dahlén G. Genotypic variation and capsular serotypes of *Porphyromonas gingivalis* from chronic periodontitis and periodontal abscesses. *FEMS Microbiol Lett* 2007 (in press).
- IV. Yoshino T, Björkner A, Dahlén G. Interaction of *Porphyromonas gingivalis* with human epithelial cells. Manuscript.

ABBREVIATIONS

Common abbreviations used in this thesis are listed according to their first appearance.

AFLP	Amplified Fragment Length Polymorphism
Arg	arginine
CFU	colony forming unit
ChP	chronic periodontitis
Cv	combined virulence
<i>fimA</i>	fimbriae gene
<i>kgp</i>	lys-specific cysteine proteinase gene
LPS	lipopolysaccharides
Lys	lysine
MABs	monoclonal antibodies
MIC	minimum inhibitory concentration
OMGS	Oral Microbiology Göteborg Sweden
OMV	outer membrane vesicles
PCR	Polymerase Chain Reaction
<i>prtC</i>	collagenase gene
RAPD	Random Amplified Polymorphic DNA
<i>rgpA</i>	arginine-specific cysteine protenase gene
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

INTRODUCTION AND BACKGROUND

1. Periodontal disease

1.1 General characteristics

Periodontal disease is the most common chronic inflammatory disorder in the tissues surrounding tooth in adult oral cavity. It is generally divided into two different disease types, “Gingivitis” and “Periodontitis” (Kinane and Lindhe, 2003). “Gingivitis” is defined as an inflammatory condition in soft gingival tissues surrounding the teeth without loss of periodontal supporting tissues, whereas “Periodontitis” refers to an inflammation in gingival tissues with loss of periodontal supporting tissues including the periodontal ligament and alveolar bone. Both conditions are induced and maintained by the dental plaque (biofilm) accumulated on the tooth surface and in the gingival pocket (subgingival plaque). Gingivitis and periodontitis are thus considered infections; however, it is not known what makes gingivitis to turn into periodontitis. The current hypothesis is that we are dealing with a subgingival microbial community that of various reasons increases its metabolic activity and starts to grow. This results in an imbalance of the host-bacterial ecosystem in the subgingival site (Marsh, 2003a).

Periodontal disease is currently classified into several forms as chronic periodontitis, aggressive periodontitis, necrotizing periodontal disease and periodontal abscess (Armitage, 1999). It is widely accepted that the two main forms of destructive periodontal disease are the chronic and aggressive forms (Kinane and Lindhe, 2003; Tonetti and Mombelli, 2003). Chronic periodontitis(ChP) is defined as an infectious disease inducing an inflammatory reaction and subsequent loss of supporting tissue and alveolar bone of the teeth if no periodontal treatment is provided. It results in periodontal pocket formation and/or gingival recession (Kinane and Lindhe, 2003). Aggressive periodontitis is recognized as a specific type of periodontitis with clearly identifiable clinical and laboratory characteristics such as “rapid attachment loss and bone destruction” and “familial aggregation” (Tonetti and Mombelli, 2003). Further features in this form of periodontitis are an elevated

proportion of a certain periodontal microorganisms e.g. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* with in subgingival flora (Tonetti and Mombelli, 2003). Periodontal abscess is also commonly found in patients with moderate or advanced periodontitis (Xin Meng, 1999). This is recognized as a localized purulent infection of periodontal tissue that may also lead to destruction of the periodontal attachment and bone (Xin Meng, 1999). All these forms of periodontitis are induced and maintained by bacteria in the subgingival plaque, today renamed “the dental biofilm”. The mechanism behind inducing and maintaining periodontal diseases is unclear and the role of various bacterial species in disease progression is currently unknown.

1.2 Dental plaque biofilms

Dental plaque biofilm is defined as the complex community of microorganisms formed on the tooth surfaces, embedded in an extracellular matrix of polymers of host and bacterial origin (Costerton *et al.*, 1987; Lawrence *et al.*, 1991).

A major advantage for the bacteria in the biofilm is protection from detrimental environmental factors such as host defense factors and antimicrobial substances including antibiotics (Costerton *et al.*, 1987; Costerton *et al.*, 1994; Wright *et al.*, 1997). The decreased susceptibility to antimicrobial agents may be due to inhibition of antimicrobial penetration into the biofilm by the extracellular polymeric substance matrix, by low metabolic activity and by changed phenotypic expression of bacterial genes (Ceri *et al.*, ; Gilbert *et al.*, 1997; Gilbert *et al.*, 2002; Kinniment *et al.*, 1996; Pratten and Wilson, ; Wilson, 1996). Biofilms can also facilitate the processing and uptake of nutrients, cross-feeding (one species providing nutrients for another), removal potentially harmful metabolic products (often by utilization by other bacteria) and development adaptive environment (reduced oxidation reduction potential) (Socransky and Haffajee, 2002).

The plaque biofilm also promotes a continuous release of bacterial surface components into the oral cavity and the gingival sulcus (Sutherland, 2001) that may result in enhanced pathogenicity of biofilm communities. Thus, the formation of the dental plaque biofilms with a complex bacterial composition is an important etiological factor in periodontal disease.

1.3 Oral bacteria in periodontal disease

It is estimated that more than 500 bacteria species can be identified within the plaque biofilm of the gingival pocket (Moore and Moore, 1994; Paster *et al.*, 2001). Possibly, 10 – 30 species may play a more critical role in the pathogenesis of periodontal disease (Socransky and Haffajee, 1994). The colonization of bacteria on tooth surfaces adjacent to the gingival margin and/or subgingival pocket is the first step in the pathogenesis of periodontal diseases.

A marked qualitative and quantitative difference between periodontal healthy and periodontitis subjects has been demonstrated (Socransky *et al.*, 1998; Ximenez-Fyvie *et al.*, 2000a; Ximenez-Fyvie *et al.*, 2000b). The predominating microorganisms isolated from the teeth and gingival sulcus of periodontally healthy individuals include mainly Gram-positive, facultatively anaerobic bacteria, and rarely Gram negative anaerobic rods (Marcotte and Lavoie, 1998).

The Gram-negative anaerobic bacteria on the other hand are found to be predominant in the subgingival microflora with increasing severity of periodontal disease (Slots, 1977; Slots and Rams, 1991). Among these Gram-negative bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* has been designated the red complex by Socransky *et al.* (1998). These red complex species are significantly predominant in the periodontal pocket and associated with periodontal progression (Socransky *et al.*, 1998; Ximenez-Fyvie *et al.*, 2000a; Ximenez-Fyvie *et al.*, 2000b). In a consensus report (World Workshop in Periodontology from the 1996), it was also suggested that *Porphyromonas gingivalis*, *Tannerella forsythia* and *Actinobacillus actinomycetemcomitans* are specific periodontal pathogens and causative agents in periodontal disease. Such a specific role for these bacterial species has not so far been proven.

2. *Porphyromonas gingivalis*

Porphyromonas gingivalis is a gram-negative, anaerobic, non-motile, asaccharolytic and black pigmented rod that form greenish-black colonies in blood agar plates (Haffajee and Socransky, 1994). Fresh clinical isolates of this organism have different colony morphologies, ranging from smooth to rough colony morphotypes (Reynolds *et al.*, 1989). In both periodontitis and healthy subjects, *P.*

gingivalis can be recovered in low frequency from the subgingival flora, tongue, buccal mucosa and tonsils and saliva (Dahlén *et al.*, 1992; Danser *et al.*, 1996; van Winkelhoff *et al.*, 1988; Zambon *et al.*, 1981). It is frequently found in purulent infection in the head and neck region (Dahlén, 2002; Iida *et al.*, 2004), endodontal infection (Haapasalo *et al.*, 1986) and periodontal abscesses (Ashimoto *et al.*, 1998).

2.1 *Porphyromonas gingivalis* in periodontal disease

P. gingivalis is frequently detected in deep periodontal pockets in adults (Ali *et al.*, 1996; Ashimoto *et al.*, 1996; Griffen *et al.*, 1998; Papapanou *et al.*, 1997; van Winkelhoff *et al.*, 2002). The frequency of *P. gingivalis* in periodontitis are estimated within the range of 60 to 100%, while it is found in 11 to 25% of healthy subjects (Ali *et al.*, 1996; Ashimoto *et al.*, 1996; Griffen *et al.*, 1998; Papapanou *et al.*, 1997; Söder *et al.*, 1993; van Winkelhoff *et al.*, 2002). In addition, it has to be pointed out that when found in healthy cases or sites *P. gingivalis* is present in low numbers, while in deep periodontal pockets the level is significantly higher. In some cases/sites *P. gingivalis* is totally predominating and thereby substantiate the over growth and “ecological catastrophe” suggested as a characteristic of the ecological plaque hypothesis (Marsh, 2003b). The presence of *P. gingivalis* has also been correlated with periodontal pocket depth (Dahlén *et al.*, 1992; Grossi *et al.*, 1995).

Another criteria for associating a pathogen to the periodontal infection is the elevated immune response against the pathogen and specific antigens. Higher serum titers of antibodies against *P. gingivalis* in periodontitis patients than in periodontally healthy have been demonstrated (Naito *et al.*, 1984; Papapanou *et al.*, 2000; Whitney *et al.*, 1992). Elimination of this bacteria from periodontal pockets can arrest further breakdown of periodontal supporting tissues (Chaves *et al.*, 2000; Renvert *et al.*, 1996; Wennström *et al.*, 1987).

In addition to the strong clinical association between *P. gingivalis* and periodontitis, this microorganism show a number of virulence factors, some of them unique among members of the oral flora, that strengthen its pathogenic capacity.

3. Virulence of *Porphyromonas gingivalis*

3.1 Virulence concept

Virulence is defined as the relative capacity of a microbe to cause disease (Slots, 1999) or to interfere with a metabolic or physiological function of the host (Holt and Ebersole, 2005). The virulence also refers to the ability of an organism to express pathogenicity (Salyers and Whitt, 2002).

To distinguish a virulent microbe from an avirulent one, the virulent are characterized by specific metabolic end-products, extracellular toxins and enzymes, the biochemical composition of cell wall and surface components and antibiotic susceptibility. All these factors participate in the ability to evade host defense mechanisms and to invade and survive in host cells and tissues (Fives-Taylor *et al.*, 1999; Holt *et al.*, 1999).

Poulin and Combes (1999) defined the concept of virulence in terms of the “virulence factors”, which are molecules or components from a microbe that harm the host. Recently, Holt and Ebersole (2005) have proposed that virulence factors have multiple functions such as 1) the ability to participate in microbe-host interactions (adhesion); 2) the ability to invade the host; 3) the ability to grow in the host cells; 4) the ability to evade/interfere with the host defense system.

P. gingivalis has a wide range of significant virulence factors such as fimbriae, capsular polysaccharide, outer membrane vesicles, hemagglutinin, lipopolysaccharides (LPS), enzyme activity and protein antigens that all potentially contribute to its pathogenicity in periodontal disease (Haffajee and Socransky, 1994).

3.2 Virulence factors in *Porphyromonas gingivalis*

3.2.1 End-products of metabolism:

The bacterial metabolic end-products (e.g. volatile short chain fatty acids, sulfur products and ammonia) can contribute to the nutritional resources and support other bacteria within biofilm, as well as toxicity to host cells (Holt *et al.*, 1999). The short-chain fatty acids such as succinate, isobutyrate and isovalerate can inhibit the function of neutrophils (Rotstein *et al.*, 1987; Rotstein *et al.*, 1989), T-lymphocytes (Eftimiadi *et al.*, 1991; Kurita-Ochiai *et al.*, 1995), phagocytes (Eftimiadi *et al.*, 1990), gingival fibroblasts (Singer and Buckner, 1981) and periodontal ligament cells

(Eftimiadi *et al.*, 1993). Hydrogen sulfide and methyl mercaptan have been detected in significant amounts in periodontal pockets (Persson, 1992). Ammonia is strongly cytotoxic to neutrophils and gingival fibroblasts (Bartold *et al.*, 1991; Niederman *et al.*, 1990; van Steenberg *et al.*, 1986). Since all these bacterial metabolites are smaller molecules than other cytotoxic factors e.g. proteases and lipopolysaccharides, they may more easily penetrate into the periodontal tissues at an increased bacterial metabolic activity and growth. An increase of excretion of metabolic waste products is consequently an important part of the virulence (Tonetti *et al.*, 1987) and, thus contributes to periodontal tissue destruction.

3.2.2 Lipopolysaccharides (LPS):

LPS are major surface components of Gram-negative bacteria and they are building up a complex consisting of polysaccharide, the core polysaccharide and Lipid A. Lipid A is the toxic part of LPS and has endotoxic activity and stimulates host inflammatory response indirectly by host derived cytokines (Bartold *et al.*, 1991; Yamaji *et al.*, 1995), and the polysaccharide chain constitutes the O-specific antigen and has also significant immunological activity (Takada *et al.*, 1992). Enterobacterial LPS can stimulate macrophage/monocytes to produce pro-inflammatory cytokines, while *P. gingivalis* is less potent (Hirschfeld *et al.*, 2001; Shapira *et al.*, 1998).

3.2.3 Capsule:

Bacterial capsules have been considered major virulence factors on the bacterial cell surface (Holt *et al.*, 1999). It is formed by a polysaccharide heteropolymer on the outer membrane of the bacterial cell (Woo *et al.*, 1979). It has various functions forming a physiochemical barrier for the cell protecting against opsonization and phagocytic host cells e.g. neutrophils (polymorphonuclear leukocytes:PMNs) and from desiccation (Chen *et al.*, 1987; Sundqvist *et al.*, 1991; Van Steenberg *et al.*, 1987). Especially, the antiphagocytic activity against host cells is important for a periodontal pathogen such as *P. gingivalis* in its penetration into the host tissue in periodontal pockets, and survive and multiply in this area.

3.2.4 Fimbriae:

The fimbriae of *P. gingivalis* are filament components of the cell surface structure with a diameter of 5 nm and a pitch of 33 nm. They are highly antigenic and show high serum IgA and IgG antibody responses (Ogawa *et al.*, 1990; Yoshimura *et al.*, 1987). The most essential role of fimbriae is the binding capacity to host cells including the oral epithelial cells, gingival fibroblasts and endothelial cells, other bacterial species, extracellular matrix protein and salivary proteins (Hamada *et al.*, 1998). Especially, the binding activity to oral epithelial cells can be the first step in its invasion and survival in host gingival tissues and thus contribute to enhance the pathogenicity of this organism. In addition, minor (short) fimbriae induce production of several cytokines from macrophages that in turn can induce alveolar bone resorption (Hamada *et al.*, 2002).

3.2.5 Extracellular proteolytic enzymes:

P. gingivalis produces a wide variety of enzymes. Of these, the Arg-X and Lys-X specific extracellular cysteine proteinases can degrade serum proteins including immunoglobulin and complement factors as well as extracellular matrix proteins (e.g. fibrinogen, laminin) and activate cytokines (e.g. tumor necrosis factor- α , interleukin-6) (Kadowaki *et al.*, 2003). This family of cysteine proteinases have been given the name “gingipains” (Curtis *et al.*, 1999). The gingipains constitute a group of cysteine endopeptidases that are responsible for at least 85% of the general proteolytic activity (Potempa *et al.*, 1997) and 100% of the “trypsin-like activity” produced by *P. gingivalis* (Potempa *et al.*, 1995). Therefore, gingipains are important virulence factors in the periodontal infection, even if the detailed role of these enzymes are not known.

3.2.6 Outer membrane vesicles (OMV):

Most gram-negative bacteria form small structures on the outer membrane surface of bacteria named “outer membrane vesicles”. This OMV are released from the outer membrane during growth (Handley and Tipler, 1986). The OMV of *P. gingivalis* may contain several virulence factors including gingipains (Marsh *et al.*, 1989).

4. Host-parasite interaction

A range of distinct microbial ecosystems are existing in the nature. The host-parasite interaction may be used to describe “an environmental adaptive process between host and microorganisms” in such ecosystems in the body. Hence, the host induces a defense response against foreign substances e.g. bacteria and their products, while the microorganisms may colonize on and invade in host tissues to survive and grow under favorable conditions.

4.1 Host-parasite interaction in periodontal tissue

Bacteria that are forming biofilms on the tooth surface normally extend down into gingival sulcus (subgingival area). At the bottom of the gingival sulcus, the gingival epithelium forms a thin lining (15-20 cell layers in coronal portion and 3-4 at the cement-enamel junction, Lindhe *et al.*, 2003). The cells of the junctional epithelium are directly exposed to bacteria and their products. The interaction of periodontopathogenic bacteria with the epithelial cells of the subgingival area, therefore, provides a chance to enter the host tissues which is a crucial step in the periodontal infection and destruction of periodontal supporting tissues (Bosshardt and Lang, 2005).

The mechanisms involved in the host-bacterial interaction in periodontal tissues are not fully understood. Oral bacteria that may cause periodontal disease are considered to produce a multiple virulence factors that all increase the ability of the bacteria to colonize, grow, invade, survive and multiply and evade the host defenses in periodontal pocket and tissues (Holt *et al.*, 1999).

The microbial invasion of host cells and tissues is the initial event in the pathogenesis of any bacteria going from a colonizing stage to be infectious. The primary ecological niche of periodontopathogenic bacteria is the gingival sulcus and periodontal pocket. *P. gingivalis* possess the ability to adhere to and invade into the gingival pocket epithelium by multimodal binding mechanisms (Houalet-Jeanne *et al.*, 2001; Lamont *et al.*, 1992; Lamont *et al.*, 1995; Madianos *et al.*, 1996; Papapanou *et al.*, 1994; Sandros *et al.*, 1994). Internalization of *P. gingivalis* into the gingival pocket epithelial cells has been considered as a critical strategy for this organism to protect itself from phagocytosis by the professional phagocytic cells e.g. neutrophils and macrophages (Lamont *et*

al., 1992; Madianos *et al.*, 1996; Sandros *et al.*, 1994). However, the invasion of *P. gingivalis* into periodontal tissues may be hampered by the continuous exfoliation of epithelial cells into gingival sulcus, and by a neutrophilic barrier that constitutes the major part of host cells in the gingival exudates (Lindhe *et al.*, 2003). The gingival fluid flow from the widened interstitial space of junctional epithelium continually transport host cells, non-adherent bacteria and its products through the gingival pocket into the oral cavity (Schroeder and Listgarten, 1997). Thus, the ability of *P. gingivalis* to grow in the subgingival, and to invade and survive within periodontal epithelium and connective tissue is suggested to be critical for their presence and association in periodontitis.

5. Virulence diversity of *Porphyromonas gingivalis*

5.1 Specific virulent clone hypothesis of *P. gingivalis*

The suggestions that some more virulent clonal types may exist among *P. gingivalis* isolates are referred to as the “*specific virulent clone hypothesis of P. gingivalis*”.

5.1.1 • Animal abscess formation by *P. gingivalis*:

A number of animal models have been used to evaluate the pathogenicity of *P. gingivalis*. The models use subcutaneous injections of bacterial cell suspensions and the capacity to form abscesses is determined. The models show the outcome of the host-bacterial interaction once the bacteria are in the connective tissue; however, it does not deal with the event of penetration on the epithelial barrier. On the other hand, the experimental abscess model in mice clearly shows abscess formation by *P. gingivalis*. Two main virulence groups have been identified, one causing mild or localized abscesses (e.g. strain FDC381) and the other causing severe and spread abscesses with risk of killing the animal due to sepsis (e.g. strain W83/W50) (Grenier and Mayrand, 1987; Neiders *et al.*, 1989; van Steenberg *et al.*, 1982; Van Steenberg *et al.*, 1987). Thus, this suggest the existence of at least two clonal types, one virulent or invasive (e.g. strain W83/W50) and one avirulent or non-invasive (e.g. strain FDC381) (Grenier and Mayrand, 1987).

5.1.2 •in vivo alveolar bone loss by *P. gingivalis*:

The ability to induce alveolar bone loss has also been investigated in animal models. Evans *et al.* (1992) reported the different ability to induce alveolar bone loss between different *P. gingivalis* strains in gnotbiotic rats. The diversity in the induction of alveolar bone loss among *P. gingivalis* strains has been evaluated in mice (Baker *et al.*, 2000). Non-invasive type of *P. gingivalis*, strain FDC381 did not induce bone loss in mice, whereas other *P. gingivalis* including invasive type of *P. gingivalis* strain W50 clearly induced bone loss, thus conforming the difference in virulence between the two strains W83/W50 and FDC381.

5.1.3 •in vitro phagocytosis of *P. gingivalis*:

Resistance to phagocytosis by mainly polymorphonuclear leukocytes (PMNs) plays a critical role for the survival of *P. gingivalis* in the periodontal tissues. Sundqvist *et al.* (1991) demonstrated a significant difference among *P. gingivalis* strains in their interaction with human PMNs. The invasive type of *P. gingivalis* e.g. strain W83, was poorly phagocytized, whereas the non-invasive type e.g. strain FDC381 was highly phagocytized. This was partly supported by Cutler *et al.* (1991) who suggested that the strain W83 resisted phagocytosis, but strain ATCC 33277 which is genotypically similar to strain FDC381 was less resistant. This again indicates that there might be a difference in virulence between the *P. gingivalis* strains on a genotype level.

5.1.4 • Serological studies in *P. gingivalis*:

Various biochemical subtype tests such as biotyping, antibiotyping and serotyping have been used to distinguish individual isolates of *P. gingivalis* (Fisher *et al.*, 1986; Laliberte and Mayrand, 1983; Notten *et al.*, 1985; Parent *et al.*, 1986). Among these techniques, serotyping has been extensively used to identify difference in pathogenicity of *P. gingivalis* isolates (Fisher *et al.*, 1986; Gmür *et al.*, 1988; Nagata *et al.*, 1991; Parent *et al.*, 1986), based on the hypothesis that difference in virulence between *P. gingivalis* isolates is due to surface components that protect the bacterial cell from phagocytosis (Grenier and Mayrand, 1987; Sundqvist *et al.*, 1991; Van Steenberghe *et al.*, 1987). Fisher *et al.* (1986) thus reported a relation between pathogenicity and *P. gingivalis* serotypes (A and B) based on cell membrane lipopolysaccharides and protein antigens. This report suggested that

serotype B strains (e.g. strain W83/W50) were more associated with pathogenicity than their serotype A strains (e.g. strain FDC381) in animal abscess model. However, the clonal structure within the two serotypes are not known.

Moreover, 6 serotypes based on capsular K-antigens have been identified among *P. gingivalis* strains from periodontitis patients (van Winkelhoff *et al.*, 1993). Laine and van Winkelhoff (1998) compared the pathogenicity between capsulated *P. gingivalis* isolates (K-antigen positive) and non-capsulated *P. gingivalis* isolates (K-antigen negative) in a mouse model and revealed that non-capsulated isolates (e.g. type strain FDC381) were less virulent/invasive than capsulated isolates (e.g. type strain W83 etc.). In a series of clinical studies of K-antigen serotypes, it was revealed that K5 and K6 serotypes were more predominant than the others while more than 50% of the *P. gingivalis* isolates were capsular non-typeable (Laine *et al.*, 1997; Van Winkelhoff *et al.*, 1999). Further studies on the adhesion capacity to the epithelial cells also demonstrated that the capsulated *P. gingivalis* strains showed significantly higher adhesion capacity to epithelial cells than non-capsulated strains (Dierickx *et al.*, 2003). However, the clonal structure within capsulated and non-encapsulated strains is not known.

5.1.5 • Virulence biotype studies based on genotyping of *P. gingivalis*:

In order to evaluate the virulence of *P. gingivalis* isolates, some attentions have recently been directed to the genetic diversities of some relevant virulence factors. Some putative virulent genes of *P. gingivalis* have been purified and cloned. In one of these studies, the prevalence of the collagenase gene (*prtC*) among 21 clinical isolates of *P. gingivalis* was evaluated by polymerase chain reaction (PCR) (Bodinka *et al.*, 1994). Of the 21 isolates of this organism, 16 isolates were shown to be positive for the presence of *prtC* using DNA hybridization with a digoxigenin-labeled *prtC* PCR product as probe, while 5 *P. gingivalis* isolates were negative. In 12 of the 16 *prtC* positive isolates, identical fragment patterns revealed by the restriction fragment analysis of the PCR products, and in remaining isolates, four distinct patterns were found. The author suggested that the presence of *prtC* may indicate a higher virulence of *P. gingivalis* isolates compared with those isolates lacking this gene.

Allaker *et al.* (1997) has identified three *rgpA* genotypes (type A - C) based on polymorphism in the Arg-gingipain A (*prpR1/rgpA*) gene catalytic domain encoding arginine-specific cysteine proteinase. The majority of the isolates (77%) from 17 chronic periodontitis subjects displayed type A *rgpA* genotype. Consequently, the author suggested that all *P. gingivalis* strains may not be equally virulent.

In addition, *P. gingivalis* fimbriae (*fimA*) gene corresponding to filament components on the cell surface has been classified into 6 genotypes based on their nucleotide sequences (Nakagawa *et al.*, 2000). *P. gingivalis* *fimA* is considered to play an important role in the colonization and invasion of the bacteria into periodontal tissues. Amano *et al.* (2000) examined the prevalence of *P. gingivalis* *fimA* genotypes (type I-V and Ib) in both periodontally healthy and periodontitis patients among Japanese, and revealed the type II *fimA* gene (66.1%) to be the most predominant in the *P. gingivalis*-positive periodontitis patients. In contrast, a majority of the periodontally healthy patients showed the *P. gingivalis* type I *fimA* gene (76.1%). The data was also supported by the result from Missailidis *et al.* (2004) and van der Ploeg *et al.* (2004). These findings may suggest that according to cell-surface components both virulent and non-virulent variants of *P. gingivalis* are existing. It can be noted that the two strains FDC381 and W83/W50 of *P. gingivalis* belongs to different *fimA* groups (Type I and IV), suggesting that *fimA* IV may belong to a more virulent clone. Moreover, *P. gingivalis* type II *fimA* genotype strains showed significantly more adhesion and invasion to the epithelial cells than other *fimA* types in an *in vitro* study (Nakagawa *et al.*, 2002). Furthermore, inflammatory relation induced by different *P. gingivalis* *fimA* genotypes was investigated in mouse abscess model (Nakano *et al.*, 2004) and showed stronger inflammatory reactions for type Ib, II and IV, in contrast to the milder reactions for type I and III.

5.2 Non-specific virulent clone hypothesis of *P. gingivalis*

The hypothesis that virulence does not associate to a certain specific virulent clone or clones is referred to as the “*non-specific virulent clone hypothesis of P. gingivalis*”.

5.2.1 • Genetic diversity studies by chromosomal DNA in *P. gingivalis*:

In order to evaluate sequence differences in chromosomal DNA of *P. gingivalis*, many molecular typing methods such as restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP), mutinous enzyme electrophoresis (MEE), random amplified polymorphic DNA (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR) and multilocus sequence typing (MLST) have been used (Ali *et al.*, 1997; Chen and Slots, 1994; Enersen *et al.*, 2006; Frandsen *et al.*, 2001; Genco and Loos, 1991; Koehler *et al.*, 2003; Loos *et al.*, 1990; Loos and Dyer, 1992; Loos *et al.*, 1993; Ménard and Mouton, 1993; Ménard and Mouton, 1995). These molecular typing methods have also provided evidence that different *P. gingivalis* strains may be distinguished at the DNA level and that the genotype structure is very heterogeneous.

The utility of AP-PCR for genetic analysis of *P. gingivalis* isolates was evaluated using 73 isolates (including one laboratory strain as reference) obtained from 72 periodontal patients (Chen and Slots, 1994). A total of 45 genotypes among the 73 isolates was identified. However, this study did not demonstrate the relation between particular genotypes and periodontal disease.

Ménard and Mouton (1995) investigated the genetic diversity of *P. gingivalis* by RAPD method on 97 human strains collected from various countries and 32 animal strains. Total 102 clonal types were identified among 129 *P. gingivalis* strains. The author suggested that the population structure of this organism is basically clonal, and that no relation was found between specific clusters of clonal types and the periodontal status of host. A similar study was carried out by using the MEE method on 88 human isolates and 12 animal strains (Loos *et al.*, 1993). This result also found a considerable heterogeneity of clonal types (78 genotypes) among 100 strains, which thus supported the findings by Ménard and Mouton (1995). Accordingly, a specific virulent genotype or clone was not found among these numerous strains of *P. gingivalis*. This result was recently supported by several studies on the clonality of *P. gingivalis* strains using the MLST method (Enersen *et al.*, 2006; Frandsen *et al.*, 2001; Koehler *et al.*, 2003).

Furthermore, the genotypes of black-pigmented anaerobes from subgingival plaque were examined using REA method and it was found that most subjects were colonized by one single genotype of *P.*

gingivalis and that identical genotypes could be present in both diseased and healthy sites (Teapaisan *et al.* 1996). Furthermore, in studies using beagle dogs, identical clonal types was also found among isolates recovered from both periodontally healthy and diseased pockets of same beagle dog. And no association between clonal type and periodontal status was found (Madianos *et al.* 1994).

5.2.2 • Virulence biotype studies based on genotyping in *P. gingivalis*:

Wittstock *et al.* (2000) has evaluated the heterogeneity of the *prtC* gene of *P. gingivalis* by PCR-RFLP. Nine different *prtC* genotypes were detected among *P. gingivalis* isolates from periodontitis subjects. Of these genotypes, four genotypes were more frequent. However, it was concluded that predominant clonal types have not been found to be associated with periodontal disease, and that all clonal types would be equally effective in colonizing the human host and inducing an infection.

The prevalence of *P. gingivalis fimA* genotypes has been investigated in Caucasians (Beikler *et al.*, 2003b). The result indicated that type I, II and IV *fimA* genotypes were found in the same frequency in Caucasian periodontitis patients and that no relationship was found between different *fimA* genotypes and severity of periodontal disease. Therefore, they concluded that there were no apparent geographic distribution of a specific virulent clone of *P. gingivalis*.

Further two different *kgp* genotypes (type I and II) based on the sequence variation of the Lys-gingipain (*kgp*) gene catalytic domain encoding lysine-specific cysteine protease have been identified by (Beikler *et al.*, 2003a). The same proteolytic activity was shown between type I and II *kgp* genotypes and no significance difference of the periodontal disease severity was also found between these two genotypes.

In conclusion, there is no clear evidence of specific virulent clones exists for *P. gingivalis* or if virulence could vary due to the expression of various genes depending on the local environmental and host factors.

AIMS

The main objective of the present thesis was the following:

- **Overall aim:**

To investigate the phenotypic and genotypic basis for the virulence properties of the species *Porphyromonas gingivalis*.

- **Specific aim:**

Paper I – To evaluate phenotypic heterogeneity of *P. gingivalis* strains from a Swedish population with periodontitis, or periodontal diseases.

Paper II – To evaluate genotypic heterogeneity among *P. gingivalis* Swedish periodontitis and periodontal abscess strains.

Paper III – To evaluate virulence genotype variations of *P. gingivalis* based on *fimA*, *rgpA* and *kgp* genes, and capsular K-antigens.

Paper IV – To evaluate *P. gingivalis* binding capacity to the human epithelial cells.

MATERIAL AND METHODS

Bacterial samples:

A total of 79 *P. gingivalis* strains including 55 fresh clinical isolates (labeled strain PgS 1-55) from 51 periodontitis Swedish subjects with deep periodontal pockets (≥ 6 mm), 8 clinical isolates from subjects with a periodontal abscess (Hafström *et al.*, 1994), 2 type strains (FDC381, W83), 6 representative K-serotype strains (HG 91 (K non-typeable), HG184(K2), HG1025(K3), HG1660(K4), HG1690(K5) and HG1661(K6)), 8 reference strains (OMGS 406 (from Kenyan periodontal pocket), OMGS 673 (from an infected necrotic root canal in a Swedish subject), OMGS 769 (from Kenyan periodontal pocket), OMGS 788 (from Kenyan periodontal pocket), OMGS 984 (from dorsum of the tongue in a Swedish subject), OMGS 2104 (from Chinese periodontal pocket), OMGS 1577 (from Japanese periodontal pocket) and OMGS 1578 (from Japanese periodontal pocket)) and two non-*P. gingivalis* strains (*Porphyromonas endodontalis* and *Prevotella intermedia*) were subjected to this series of studies (for details see paper I - IV) (Table. 1).

Strain designation	Type of infection	Country	Study
S 1-55	Periodontitis	Sweden	I, II, III [§] , IV ^{§§}
A 1-8	Periodontal abscess	Sweden	I, II, III [§] , IV ^{§§}
Reference W83*	Unknown	Sweden	I, II, III, IV
Reference FDC 381**	Periodontitis	U.S.A	I, II, III, IV
OMGS769***	Periodontitis	Kenya	I, II, III
OMGS 788	Periodontitis	Kenya	I, II, III
OMGS 984	Dorsum of tongue	Sweden	I, II, III
OMGS 673	Endodontic infection	Sweden	I, II, III
OMGS 1577	Periodontitis	Japan	II, III
OMGS 1578	Periodontitis	Japan	II, III
OMGS 406	Periodontitis	Kenya	II, III
OMGS 2104	Periodontitis	China	II, III
HG 91****	Periodontitis	The Netherlands	II,
HG 184	Periodontitis	The Netherlands	II,
HG 1025	Periodontitis	The Netherlands	II,
HG 1660	Periodontitis	The Netherlands	II,
HG 1690	Periodontitis	The Netherlands	II,
HG 1661	Periodontitis	The Netherlands	II,
<i>P. endodontalis</i> ^a	Endodontic infection	Sweden	I
<i>P. intermedia</i> ^b	Periodontitis	Sweden	I, II

Table. 1 Clinical and laboratory strains of *Porphyromonas gingivalis* for study I-IV.

*Kindly provided by G. Sundqvist, Department of Endodontics, Umeå University, Sweden. **Obtained from Forsyth Dental Center. ***Strains from Oral Microbiology, Göteborg, Sweden (OMGS). ****Obtained from Oral Microbiology, ACTA, Amsterdam, the Netherlands. §*Porphyromonas gingivalis* S28 not included. §§ Seventeen strains (S1, S3, S4, S10, S11, S24, S27, S31, S39, S45, S52, A1, A3, A5, A6, A7 and A8) only used

Phenotype characterization

Gas-liquid chromatography (Paper I):

Bacterial metabolic products in peptone yeast medium with 1% glucose (PYG) were evaluated using gas-liquid chromatography (Sigma 2B, Perkin-Elmer, Norwalk, Conn., equipped with a flame ionization detector) as outlined in the Virginia Polytechnic Institute (VIP) manual (Holdeman *et al.*, 1975). The glass column of the chromatography was packed with 5% AT 1000 (Altech Associates Inc., Deerfield, IL) on chromosorb GHP 100/120 mesh (Johns-Manville, Dever Co). The carrier gas was nitrogen (30ml/min), the injection port temperature 150°C and the oven temperature 120°C. One-microliter of the ether extracted or methylated samples according to Holdeman and Moore (1975) was used, and the results were compared with standard solutions of volatile fatty acids.

Biochemical tests (Paper I):

The peptone-yeast medium broth (Becton Dickinson) was used as the basal medium for analyses of fermentation of carbohydrates and derivatives by *P. gingivalis* strains. The preparation and inoculation of fermentation tubes were carried out according to the Virginia Polytechnic Institute manual.

Enzyme profiles (Paper I):

The API-ZYM colorimetric kit system (API System, La Balmes les Grottes, Montalieu-Vercieu, France) for detection of enzymes was used according to the manufacturer's directions. Color reactions were read from grade 0 to 5, whereby 0 indicates no enzyme activity, 1 and 2 weak activity and 3-5 indicate strong, significant enzyme activity.

Antibiotic susceptibility (Paper I):

Both the disc-diffusion (for primary screening) and the agar plate dilution (for minimum inhibitory concentration (MIC) determination) methods were used for antibiotic susceptibility test. Susceptibility was tested to the following antibiotics: penicillin-G, ampicillin, isoxapenicillin, tetracycline, clindamycin, kanamycin, erythromycin, metronidazole, tinidazole and oxytetracycline (for details see paper I).

Sodium dodecyl sulphate-polyacryamide gel electrophoresis (SDS-PAGE) whole protein profiling (Paper I):

Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) was performed in a mini-protein unit (Bio-Rad Laboratories, Sundbyberg, Sweden) at 200 V for 45 min by using a vertical 0.75-mm-thick slab gel containing 7.5% (weight/weight) polyacrylamide. Bacterial samples were prepared by whole-cell sonications at 50 W for 1min. The preparations were performed by heating with an SDS sample buffer at 100 for 5 min. After electrophoresis, the gel was stained with Coomassie brilliant blue.

Monoclonal antibodies (MAbs) serotyping (Paper I):

MAbs serotyping was performed by indirect immunofluorescence. Serotype B was defined by a

positive reaction with MAbs 50BG2.1, while serotype A strains reacted with 60BG1.3 or 48BG1.1 only (for details see paper I).

Capsular K serotyping (Paper III):

Capsular serotyping of the *P. gingivalis* isolates was performed by double immunodiffusion using polyclonal antisera raised against the K1 to K6 type strains. Immunodiffusion was carried out in 1.0 % agarose (Sigma Chemical Co., MO, type 1, low EEO) in 50mM Tris-HCl buffer (pH 7.6). 15 µl of undiluted antiserum and 15 µl of antigen were allowed to precipitate for 48h at room temperature.

Genotype characterization

Amplified Fragment Length Polymorphism (AFLP) of whole chromosomal DNA (Paper II):

Twenty-five nanograms of DNA templates for AFLP were prepared. Briefly, purified DNA was digested and ligated simultaneously with PstI (New England Biolabs Inc., Beverly, MA), MseI (New England Biolabs Inc.), PstI-O adapter, Mse-C adapter and T4 DNA ligase (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 4 h. A Texas Red fluorescent labeled PstI-O primer (Isogen Bioscience, Bithoven, the Netherlands) and unlabeled Mse-C primer were used for DNA amplification, which was performed in a Gene- Amp PCR System 9700 thermal cycler (Perkin Elmer, Boston, MA). Fluorescent amplified fragments were separated on a denaturing polyacrylamide gel (RapidGelXL-6%; Amersham Life Science, Cleveland, OH) according to the manufacturer's instructions in a Vista 725 automated DNA sequencer (Amersham Life Science, Cleveland, OH) (for details see paper III).

Random Amplified Polymorphic DNA (RAPD) whole chromosomal DNA profiling (Paper II):

The RAPD amplification reaction was performed in a total volume of 25 µl, consisting of 2.5 µl of 10×Stoffel Buffer, 0.4 mM of dNTPs, 3U AmpliTaq DNA polymerase, Stoffel Fragment (Applied Biosystems, CA, USA), 2 µM primer (10 µM) (USbiological, MA, USA), 4 mM MgCl₂, and 100 ng

of DNA template using a PTC-100 thermal controller (MJ Research, Watertown, MA, USA) (for details see paper III).

***P. gingivalis fimA* genotyping (Paper III):**

The determination of *fimA* genotypes was performed. The PCR amplification reaction was performed in total volumes of 25 µl, consisting of 2.5 µl of 10×PCR Buffer II, 0.2 mM of dNTPs, 3U AmpliTaq Gold DNA polymerase, (Applied Biosystem, Foster City, CA, USA), 0.8 µM each primer, 4 mM MgCl₂, and 100 ng of DNA template using a PTC-100 thermal controller (MJ Research, Watertown, MA, USA). The PCR products were visualized by 1 % agarose gel with ethidium bromide (1 µl/ml) under UV light (for details see paper III).

***P. gingivalis kgp* genotyping (Paper III):**

The determination of *kgp* genotypes were performed by PCR. After amplification of *kgp* gene, the PCR products were subjected to a restriction digestion with *Tru9 I* (*Mse I*) restriction enzyme (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. Then, the digested PCR products were visualized by 1 % agarose gel with ethidium bromide (1 µl/ml) under UV light.

***P. gingivalis rgpA* genotyping (Paper III):**

The determination of *rgpA* genotypes was performed. The PCR amplification reaction was performed in total volumes of 25 µl, consisting of 2.5 µl of 10×*Pfu* polymerase Buffer with MgSO₄, 0.2 mM of dNTPs, 3 U *Pfu* DNA polymerase (Promega, Madison, WI, USA), 0.8 µM each primer and 100 ng of DNA template. The PCR products were subjected to a restriction digestion with *RsaI* restriction enzyme (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The digested PCR products were visualized by 4.0 % agarose gel with ethidium bromide (1µl/ml) under UV light (for details see paper III).

Host - bacterial interaction

Bacteria-KB interaction by binding and invasion (Paper IV):

The *P. gingivalis* interaction assay, was performed in triplicates. KB cells were transferred to 24-well plates at a density of approximately 10^5 cells/well, resulting in confluent cultures 24 hours later. The KB cell layers (10^5 cells/well) were mixed with 500 μ l of the microbial suspension of 2.5×10^6 cells (bacteria : cells relation 25:1), washed in PBS and incubated at 37°C for 90 min. 19 different *P. gingivalis* strains was used in the assay. Non-adherent bacteria were removed by washing three times with PBS. Cell-associated bacteria (e.g. surface binding and intra cellular, invading bacteria) were quantified after lysis of the KB cell layers in 1 ml of distilled water and subsequent plating on agar. Internalized bacteria estimated as invaded bacteria to in KB cells were assessed in parallel experiments, after antibiotic application (500 μ g/ml metronidazole for 2 hours) in order to kill extra cellular bacteria.

RESULTS

Phenotypic properties of *P. gingivalis* (Paper I and III):

Colony morphology

Forty-four of the 55 clinical isolates of *P. gingivalis* and 4 reference strains showed smooth (S) colony. 10 clinical isolates were identified as rough (R) and were described also as strongly adherent to the agar surface. Additionally, three clinical strains could be described as semi-rough (SR).

There was no relation between the R and SR growth patterns and pigmentation. All eight strains from abscesses had smooth colony morphology.

Biochemical reactions

All *P. gingivalis* strains, including the type strains, were negative for nitrate reduction, showed a positive reaction for indole and gelatinase and most strains could proteolyse milk. All isolates produced phenyl acetic acid.

API-ZYM

All *P. gingivalis* strains showed positive alkaline and acid phosphatase and all revealed a positive trypsin and N-acetyl-glucosaminidase reaction. Other enzyme activities were weak or absent.

Antibiotic susceptibility

All strains of *P. gingivalis* were sensitive and gave wide inhibition zones upon exposure to penicillin G, tetracycline, ampicillin, clindamycin, metronidazole, tinidazole and erythromycin using the antibiotic disc method.

Generally, all *P. gingivalis* strains tested showed an overall susceptibility to all tested antibiotics except for kanamycin, for which all strains showed a susceptibility of 100 µg/ml or more.

SDS-PAGE protein profiling

P. gingivalis isolates were divided into six major protein groups (Groups Ia to IIc) based on protein banding patterns. No relation to MAb serotypes was found.

MAbs serotypes

All *P. gingivalis* strains reacted positively with MAbs60BG1.3 or 48BG1.1 (Fig. 1). Three strains W83, PgS 3 and PgS 10 reacted positively with 50BG2.1, indicating their identity as serotype B. Among the eight abscess strains, three strains (37%) were identified as serotype B.

Capsular serotypes

Altogether, 36% of the isolates were typeable for the capsular K-antigen (Fig. 1). 23% of the isolates belonged to the predominant K6 serotype. Among these strains, four and five strains were either type II:I:A or II:II:A Cv genotypes, respectively. The *P. gingivalis* serotype K3 was not detected among the tested strains. 64% of the Swedish *P. gingivalis* isolates showed a negative reaction for capsular K-antigen. Interestingly, both S3 and A7, showing the same Cv genotype as the virulent *P. gingivalis* strain W83, revealed the K1 serotype. However, no relationship between a certain specific virulence genotype or Cv genotype and capsular K-serotype was found.

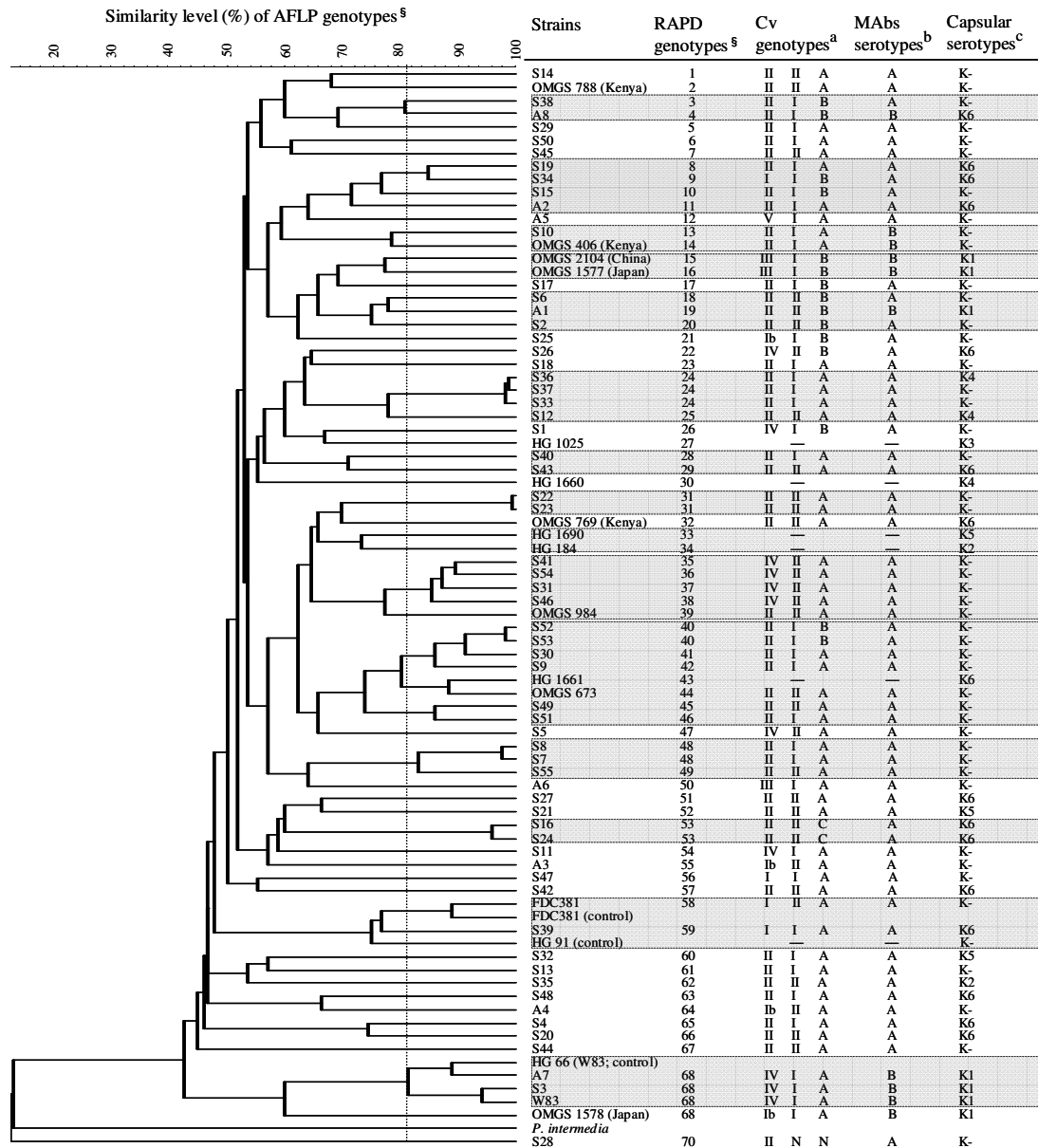


Figure 1. The summary of the heterogeneity of *P. gingivalis* strains by phenotyping (Paper I and III, b and c) and genotyping assays (Paper II and III, § and a). A dot line shows 81% of AFLP genotypes similarity. A gray rectangle described clusters by 70% similarity.

Genotypic properties of *P. gingivalis* (Paper II and III):

AFLP genotypes

A dendrogram of AFLP genotyping of 78 *P. gingivalis* strains, including reference *P. gingivalis* strains and one *P. intermedia* strain, is shown in Fig. 1. The majority of the *P. gingivalis* strains, with the exception of strain S28, fell into a single group at a similarity level of more than 45%. Strain S28 was clearly different from the other *P. gingivalis* strains. When all the *P. gingivalis* strains were clustered at a similarity level of 70%, *P. gingivalis* strains revealed 15 (I–XV) clusters. Most of the clusters were provided by small groups consisting of only two or three clones. Duplicate AFLP genotype patterns of strain FDC 381 and W83 were used to evaluate the similarity level within the same strain (Fig. 1). The FDC 381 and W83 had similarities of 89% and 81%, respectively. A similarity of 81% as the cut-off point was therefore used to detect same or similar AFLP genotype profiles. Consequently, 62 AFLP genotypes were detected among the 78 *P. gingivalis* strains.

RAPD genotypes

The RAPD profiles of each strain were compared with the banding patterns of their counterparts and strains with the same or similar banding patterns were regarded as identical genotypes. The 78 *P. gingivalis* strains were classified into 70 RAPD genotypes.

The majority of *P. gingivalis* strains showed distinct single RAPD banding patterns and provided an extensive genetic heterogeneity (data not shown). Thirteen strains were grouped into six identical RAPD genotypes. Of these, two strains S3 and A7 revealed genotypes identical to that of the type strain W83 (Fig. 1).

Moreover, four pairs of identical genotypes (e.g. S7 and S8, S22 and S23, S36 and S37, S52 and S53) were obtained each from two deep periodontal pockets in four subjects. Another three pairs of identical genotypes were isolated from unrelated subjects.

AFLP/RAPD-matched genotypes

The AFLP and the RAPD cross-matched identical genotypes that showed the same or similar genotypes with the two genotyping methods were assessed to disclose more proper diversity of the *P.*

gingivalis genotypes. A total of 70 AFLP/RAPD matched genotypes were found among the 78 *P. gingivalis* strains. In addition, 56 AFLP/RAPD genotypes were found among 63 clinical isolates from Swedish subjects. The majority of Swedish isolates showed rather different individual genotypes and diversity. Six identical genotypes were also detected in AFLP/RAPD genotype analysis, in accordance with the observation from RAPD profiling. Interestingly, strains S3 and A7 showed clonal genotype similarity with the type strain W83 in the combined AFLP/RAPD profiling. No identical genotype corresponding to type strain 381 was among the Swedish clinical isolates.

***P. gingivalis fimA* genotypes**

Among *P. gingivalis* isolates, type II *fimA* genotype was predominant (71%). Type IV *fimA* was the second most prevalent (16.1%), while the other *fimA* genotypes were detected only in a few strains (1.6–4.8%).

***P. gingivalis kgp* genotypes**

Type I and II *kgp* genotypes were found at a similar level (56.5% and 43.5%, respectively).

***P. gingivalis rgpA* genotypes**

Of the three genotypes found in 75.8% of isolates, type A *rgpA* was the most frequent, while type B and C *rgpA* genotypes were detected in 21% and 3% of the isolates, respectively.

Combined virulence (Cv) genotypes

A total of 18 Cv genotype combinations of *fimA*, *kgp* and *rgpA* virulence gene morphotypes were identified among *P. gingivalis* strains, including reference strains, tested (Fig. 1). The majority of the clinical isolates (53.3%) was of either type II:I:A, or II:II:A Cv genotype (31% and 22.5%, respectively). The genotypes II:I:B and IV:II:A were detected in 10% and 8% of the isolates, respectively. Strain W83 was classified as type IV:I:A together with three other isolates, two of which were K1 strains. No Cv genotype (I:II:A) identical to strain 381 (avirulent strain) was found.

Eight isolates representing four pairs from two periodontal pockets each of four patients were of the same Cv genotype.

Virulence determination of *P. gingivalis* (Paper IV):

Bacteria-KB cell interaction

The cell-association including bacterial surface (binding) adherence and invasion into the KB cells was studied. The number of bacteria associated with the KB cells was expressed as a mean value of logarithm number (CFU/10⁵ cells). All *P. gingivalis* adhered to and invaded into KB cells in low numbers (approximately 0.4/epithelial cell in total cell associated and 0.1/epithelial cell invaded). However, the cell binding and invasion properties varied among the *P. gingivalis* strains, ranging from 2.8 (SD ±0.2) to 5.2 (SD ±0.06) (log CFU/well), and from 2.4 (SD ±0.31) to 4.6 (SD ±0.06) (log CFU/10⁵), respectively. Significant higher binding and invasion were found for capsular K non-typeable *P. gingivalis* strains compared to the capsulated strains (K1 and K6) ($P<0.01$). Furthermore, the cell association for MAbs serotype A strains were significantly higher than MAbs serotype B strains ($P<0.01$). However, there was no relation between the binding capacity and of any of the specific virulence genotypes (*fimA*, *kgp* and *rgpA*).

MAIN FINDINGS

§ *P. gingivalis* strains showed a strong homogeneity in biochemical tests and antibiotic susceptibility. The majority of *P. gingivalis* strains displayed monoclonal antibodies (MAbs) serotype A, while serotype B was unusual among *P. gingivalis* strains. Furthermore, ten of the 55 investigated strains showed a rough colony morphology, while the rest was smooth (Paper I).

§ *P. gingivalis* isolated from Swedish subjects with periodontitis and periodontal abscess exhibited a wide variety of genotypes with weak clustering pattern. No predominant genotype at the whole chromosomal DNA level was present among these *P. gingivalis*. However, two strains showed to be of the same genotype as the virulent strains (Paper II).

§ Chronic periodontitis is not associated with a particularly virulent genotype of *P. gingivalis*. Eighteen genotype combinations were found and >50% of the isolates were a combination between *fimA* II, *rgpA* A and *kgp* I or II. A highly virulent genotype (e.g. strain W83) of *P. gingivalis* can be detected infrequently in periodontitis subjects (Paper III).

§ All strains showed a low binding capacity to host epithelial cells. Capsulated *P. gingivalis* compared to non-encapsulated strains displayed an even significantly lower binding capacity to host cells. No significant difference in binding and invasion was found between specific virulent genotypes. Thus, the two major virulence groups within *P. gingivalis* were mainly related to the presence/absence of a capsule structure of this organism (Paper IV).

GENERAL DISCUSSION

The present works (study I-IV) have demonstrated a number of phenotypic and genotypic characteristics of *P. gingivalis* with relevance to virulence. The studies were conducted in the view of two main hypothesis for explaining the relation between *P. gingivalis* isolates and periodontal infection. On one hand, there may exist specific virulent clones that are more pathogenic which explains the disease progression and severity in some patients in contrast to the majority of strains that are less virulent and is present in healthy or non-progressing sites. On the other hand *P. gingivalis* may exist in a non-clonal structure/or low clonal structure with little difference in virulence on a genotype level and variations in virulence is due to the phenotypic expression induced by environmental and host factors. Variation in virulence within the same strain could also be due of genetic rearrangements and recombinations (Holt *et al.*, 1999).

Many studies have tried to characterize more virulent types of this organism by the phenotypic expression of various virulence factors e.g. colony morphotypes, biochemical activity, enzyme production, antibiotic susceptibility, fimbriae, antigenic properties, capsule formation and adherence to various host cells (hemagglutination, epithelial cells, neutrophils, fibroblasts etc) (Holt *et al.*, 1999). This study confirmed (Paper I) a considerable homogeneity for *P. gingivalis* isolates from periodontitis patients concerning phenotypic characteristics biochemically, enzymatically and by antibiotic susceptibility however heterogeneity when it comes to colony morphology, antigenic properties, capsule formation and cell binding capacity (erythrocytes, epithelial cells and neutrophils) (Paper I, III, IV, unpublished observations). Since many of these characteristics are preserved phenotypically, it may be reasonable to consider a genetic basis for the diversity of virulence within *P. gingivalis* (The reference strain W50/W83, was isolated in the 1960ies and is still shown virulent in various laboratories). Hence, it has also been suggested that phenotypic tests cannot generally distinguish genetically different or unrelated genotypes of the same species (Loos *et al.*, 1993).

Many Gram-negative bacteria show different colony morphotypes and most of them can grow in smooth (S) or rough (R) forms on agar plates under laboratory conditions (Lüderitz *et al.*, 1966). R-forms of bacteria are occurring due to several reasons. LPS is a major component of the outer membrane of Gram-negative bacteria and R-forms have an incomplete LPS side chain (Hull, 1997). Rough colony strains are thus more susceptible for compliment (Lindberg *et al.*, 1975; van Dijk *et al.*, 1981), lysozyme and lactoferrin. R-forms are generally non-capsulated and suggested to be more easily phagocytized and killed by the neutrophils (Holt *et al.*, 1999). In some bacterial species R-forms express fibrillar appendages which alter the binding capacity to host cells (Kremer *et al.*, 2000). In the present thesis only one R-strain was tested for binding and invasion to epithelial cells and was binding to the same degree as the other non-capsulated strains (Paper IV). Furthermore, none of the 8 *P. gingivalis* isolates from periodontal abscesses were rough (Paper I). Thus smooth strains are likely to be more virulent than rough strains, however it is not possible at this stage to conclude if that is due to the capsule or complete LPS or both.

Two antigenically distinct MAbs serotypes (serotype A and B) were suggested by Gmür *et al.* (1988). The antigenicity for MAbs is based on different cell surface components including LPS that sometimes is complexed with protein. MAbs serotypes coincide, if not completely, with the virulence groups causing spread (e.g. serotype B) and localized (e.g. serotype A) subcutaneous abscess formation in mice (Fisher *et al.*, 1986; Gmür, 1995). The SDS-PAGE pattern of cell wall proteins could not disclose a distinct difference between serotype A and B strains (Paper I). MAbs serotypes did not correlate either to colony morphology of *P. gingivalis* strains (Paper I). Thus, it is still unclear whether the rough strains are antigenically different from the smooth strains. However, the MAbs serotypes seems to be related to the capsule since three of four serotype B isolates and the strain W83 belonged to the capsulated K-antigen group I.

Currently, six serotypes (K1-K6) and K negative isolates have been identified based on capsular K-antigens (Laine *et al.*, 1996; van Winkelhoff *et al.*, 1993). The capsule morphology has been observed by electron microscope (Laine *et al.*, 1996). K1-K6 *P. gingivalis* strains exhibited a clear

extracellular capsule-like structure, while K antigen negative strains revealed a thin extracellular layer probably representing the electron dense outer membrane (Listgarten and Lai, 1979). However, it was not possible to correlate the presence of the capsule or the capsular antigens to a certain genotype (Paper II and III). A higher virulence of capsulated *P. gingivalis* has been demonstrated by subcutaneous abscess formation in mice (Laine and van Winkelhoff, 1998). Possible explanation of this phenomenon may be due to antiphagocytic mechanism (Watanabe *et al.*, 1992) due to the expression of cell surface molecules that participate in adhesion as well as that antigens may be hidden by the extracellular capsular structure. Furthermore, a capsule may also constitute a good barrier to protect the bacteria from the intra-cellular killing by the neutrophils. For this reason, capsulated microorganisms may escape host defence cells and may more easily invade and spread into host tissues seen by the invasive strains in the animal abscess models. Non-invasive strains are more easily phagocytized by the neutrophils than invasive strains (Miyabe *et al.*, 2004). On the other hand it was also reported in the latter paper that PMN cell viability was lower using invasive compared to noninvasive. Fifty percent of isolates obtained from periodontal abscess that may indicate a more aggressive form of the periodontal infection, was found ($P<0.05$) to be capsulated (Paper III) in contrast to 29% in chronic periodontitis.

Conclusively, it is not known whether the bacterial invasiveness is due to the capsule or not or more likely is only one piece in the virulence pattern of the more virulent and invasive strains. On the genotype level it was not possible to relate any of the phenotypic differences to specific genes or clones. On the other hand, two *P. gingivalis* strains were found in the present study to be of the same genotype as the virulent/invasive strain W83. These two were both isolated from aggressive forms of periodontitis and show that there may exist some virulent clones of *P. gingivalis* in the populations.

Considerable whole chromosomal genetic heterogeneity of *P. gingivalis* has been reported in sample from wide geographical locations (Ali *et al.*, 1997; Loos *et al.*, 1993; Madianos *et al.*, 1994; Menard and Mouton, 1993) as well as more narrow locations (Koehler *et al.*, 2003). These studies could not find any predominant genotype of *P. gingivalis* that may be associated to periodontal disease. Thus, Loos *et al.* (1993) concluded that all *P. gingivalis* may have an equal potential to cause periodontal disease. Further studies using specific virulence gene showed that no predominant genotypes were

detected within *prtC*, *fimA* and *kgp* genes polymorphism (Beikler *et al.*, 2003a; Beikler *et al.*, 2003b; Wittstock *et al.*, 2000). These findings that all isolates have potentially equal virulence are referred to “*non-specific virulent clone hypothesis of P. gingivalis*”.

Type II *fimA* of *P. gingivalis* has been predominantly detected in periodontal pockets. It was also demonstrated that type II *fimA* strains disclosed greater inflammatory reactions compare to type I strains that represented non-invasive *P. gingivalis* strain (e.g. strain FDC 381) (Amano *et al.*, 2000; Nakano *et al.*, 2004). Hence, it was speculated that the type II gene was associated with more virulent types of *P. gingivalis* that would support “the *specific virulence hypothesis of P. gingivalis*” and that certain more virulent clonal types may exist in this species. However, since the virulence of microorganisms is due to multifunctional factors (Holt *et al.*, 1999), it is likely that the variation in genes related to virulence is more complex. The observation in Paper III on the combined specific virulence gene polymorphism of *P. gingivalis*, obviously revealed a genetic variation without effect on the phenotypic expression of fimbriae or the binding characteristics to epithelial cells (Paper III and IV). Similarly, the variation in the gingipain coding genes *rgpA* and *kgp* did not influence on binding to epithelial cells. Furthermore, the same *P. gingivalis* strains showed a great variety in the whole chromosomal genotype. Thus, for *fimA*, *rgpA* and *kgp* genes and the phenotypic outcome in fimbriae and gingipain production our findings support the “*non-specific virulent clone hypothesis*”.

Adhesion, invasion and penetration of microorganisms through the epithelial barrier of the periodontal pocket are crucial events in the infection progress. Two different pathways are suggested for this process. One pathway concerns bacterial invasion through adhesion and engulfment of the bacteria by the viable epithelial cells of the junctional epithelium of the periodontal pocket. The advantage for the bacteria is that it leads to a protection from host defense factors (antibodies, complement) and phagocytic cells (neutrophils). It also gives the bacteria the possibility to multiply and penetrate into next cell (Houalet-Jeanne *et al.*, 2001; Lamont *et al.*, 1992; Lamont *et al.*, 1995; Madianos *et al.*, 1996; Papapanou *et al.*, 1994; Sandros *et al.*, 1994). However, it should be noted that the binding and engulfment of bacteria into the epithelial cells also is an important host defence mechanism (Schroeder and Listgarten, 1997). On all epithelial surfaces of the body desquamation

continuously eliminate adhering and engulfed bacteria, a mechanism that is also working in the periodontal pocket and explains why the invasion by this route may occur only infrequently.

The other pathway is by the inter-epithelial route penetrating by growth or motility through the junctional epithelial barrier. If the former mechanism is prevailing, the binding capacity of the microorganism to epithelial cells should be regarded as a virulence mechanism for invasion. If the latter is prevailing, binding capacity is not a critical factor and non-binding bacteria would more easily penetrate through the barrier than the binding. On the other hand, extracellular bacteria need protection from host defense factors and phagocytic cells e.g. neutrophils. This can be achieved by capsule formation, or release of proteolytic enzymes, leucotoxin etc.

Consequently, binding capacity within *P. gingivalis* to the epithelial cells can be two-sided. Compared to many other bacterial species in the subgingival plaque the binding of *P. gingivalis* to epithelial cells is considered low (Paper IV)(Deshpande *et al.*, 1998; Munemasa *et al.*, 2000; Papaioannou *et al.*, 2003; Quirynen *et al.*, 2001). However, the binding capacity was found to be significantly higher for the non-capsulated *P. gingivalis* strains (Paper IV)(Dierickx *et al.*, 2003). This is in line with other bacterial species e.g. *Streptococcus pneumoniae*, *Streptococcus suis* and *Haemophilus influenzae* (Benga *et al.*, 2004; Dierickx *et al.*, 2003; Hammerschmidt *et al.*, 2005; St Geme and Falkow, 1991) which all show a higher binding ability for the non-capsulated variants. In addition, Nakagawa *et al.* (2002) and Inaba *et al.* (2006) have displayed a higher adhesion and invasion capacity to the epithelial cells of *fimA* II strains than other *fimA* types. However, contrasting findings implying no relationship between the binding capacity and tested *fimA* types, was found by Umeda *et al.* (2006). Furthermore, two strains of *P. gingivalis* carrying the same *fimA* genotypes exhibited undoubtedly different invasion capacities (Dorn *et al.*, 2000). The study IV supported the findings by Dorn *et al.* (2000) and Umeda *et al.* (2006) that non-capsulated *P. gingivalis* strains exhibited higher binding and invasion capacity than capsulated strains to epithelial cells. A possible explanation to this finding was that the extra-cellular capsule-like structure may cover the adhesion molecules including the fimbriae, and also suggest that the capsule have several functions for the bacteria in the host-parasite interaction. Additionally, current unpublished data using same *P. gingivalis* strains as in study IV showed a significantly higher binding capacity ($P<0.01$) to neutrophils than capsulated strains (Yoshino *et al.*, 2007 (unpublished)). This confirms previous

studies that non-capsulated *P. gingivalis* is less resistant to phagocytosis compared to the capsulated strains (Cutler *et al.*, 1991; Haapasalo *et al.*, 1989; Sundqvist *et al.*, 1991; van Steenberg *et al.*, 1987). Thus, invasiveness of the *P. gingivalis* through the pocket epithelial barrier inter-cellularly may be mainly related to the presence of a capsule.

Concluding remark

P. gingivalis isolates from Swedish periodontal disease cases express a considerable homogeneity in most phenotypic characteristics, although variations were found in colony morphology and MABs and capsular antigen types. On the genotype level a considerable heterogeneity was found both at whole chromosomal level as for specific virulence genes. The studies support that there is generally a non-clonal structure of *P. gingivalis* although some specific virulent clones might be found infrequently in periodontitis. A capsule seems to be of particular importance for *P. gingivalis* pathogenicity.

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