Oral commensal *Prevotella* species and *Fusobacterium nucleatum*: Identification and potential pathogenic role.

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To my family

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ABSTRACT

The heterogeneous group of the Gram-negative anaerobes constitutes a large part of the indigenous oral microbiota. The present study exploited a variety of phenotypic and molecular methods for identification, taxonomic classification, investigating population dynamics, and tracing transmission of individual clones of selected Gram-negative oral anaerobes.

The main phenotypic properties used in identification of bacteria are various biochemical tests for bacterial metabolism. For identifying *Porphyromonas gingivalis* and differentiating lactose-fermenting species from non-fermenting species rapid phenotypic screening was adequate. Commercial identification kits tested failed to improve the level of identification achieved with the phenotypic screening. Only 16S rDNA PCR method could differentiate *Prevotella intermedia* and *Prevotella nigrescens*. PCR increases the reliability of identification of a range of Gram-negative anaerobic bacteria.

The substantial natural variability of glycoproteins and glycolipids on the surface of erythrocytes makes them a convenient model for evaluating the specificity of bacterial adherence. A hemagglutinating variant of *Prevotella melaninogenica* appeared fimbriated when viewed in electron microscope. The strength of *P. melaninogenica* hemagglutination was, however considerably less than that of *P. gingivalis*. The hemagglutinating agent on *P. melaninogenica* seemed to be a protein, which could be separated from the cell and bound to lactose, galactose, and raffinose-containing carbohydrates on the erythrocytes. As this potential virulence factor of *P. melaninogenica* is of a significantly lower magnitude than that of major periodontal pathogens, this hemagglutinating variant is, at most, scarcely pathogenic.

RFLP analysis of PCR-amplified 16S rDNA, using combinations of 3 or more tetrameric restriction enzymes, is an appropriate technique for differentiation and characterization of microorganisms. The hemagglutinating strains did not form a homologous group inside the *Prevotella* genus, as viewed by PCR-RFLP results from a combination of 5 tetrameric enzymes, but fell into 3 distinct clusters.

AP-PCR typing, using more than one primer has proved to be a simple, rapid, and reliable method for fingerprinting bacteria. AP-PCR typing with a combination of up to 4 primers

revealed a wide genetic diversity within the *Fusobacterium nucleatum* populations in infants with up to 7 AP-PCR types simultaneously detected in each sample. This high clonal heterogeneity combined with frequent turnover of clones might allow the species to escape the host immune response, and persistently to colonize the oral cavity. Strain turnover rate was high during the first year of life, but then persistent clones were increasingly found. In 11 of 12 infants examined, AP-PCR types persisted for up to one year.

In 5 of 8 infants with experience of acute otitis media, identical AP-PCR types were found between the nasopharyngeal and salivary isolates. Since anaerobes seem to be only transiently present in the nasopharynx and salivary contamination of the nasopharyngeal samples could be excluded, this indicates that the source of nasopharyngeal anaerobes was the oral cavity and saliva the transmission vehicle.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-V):

I. Haraldsson G, Holbrook WP. Identifying clinically important Gram-negative anaerobes from the oral cavity. Eur J Oral Sci 1999; 107: 429-436.

II. Haraldsson G, Holbrook WP. A hemagglutinating variant of *Prevotella melaninogenica* isolated from the oral cavity. Oral Microbiol Immunol 1998; 13: 362-367.

III. Haraldsson G, Meurman JH, Könönen E, Holbrook WP. Properties of hemagglutination by *Prevotella melaninogenica*. Anaerobe 2005; in press.

IV. Haraldsson G, Holbrook WP, Könönen E. Clonal persistence of oral *Fusobacterium nucleatum* in infancy. J Dent Res 2004; 83: 500-504.

V. Haraldsson G, Holbrook WP, Könönen E. Clonal similarity of salivary and nasopharyngeal *Fusobacterium nucleatum* in infants with acute otitis media experience. J Med Microbiol 2004; 53: 161-165.

In addition, some unpublished data are presented.

ABBREVIATIONS

ANOVA	analysis of variance
AOM	acute otitis media
AP-PCR	arbitrarily-primed polymerase chain reaction
bp	base pairs
CAAM	carbobenzoxy-L-arginin-7-amino-4-metylcoumarin amideHCl
DNA	deoxyribonucleic acid
FinOM	Finnish Otitis Media cohort study
IgA1	immunoglobulin A1
ITS	internally transcribed spacer
MUG	4-methylumbelliferyl-β-D-galactoside
NP	nasopharyngeal swab
NPA	nasopharyngeal aspirate
OD	optical density
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction restriction fragment length polymorphism
PFGE	pulsed-field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
sIgA	secretory immunoglobulin A
spp	species
TAE	tris acetate EDTA
TBE	tris borate EDTA
UPGMA	unweighted pair-group method with arithmetic averages
UV	ultraviolet

INTRODUCTION

The indigenous microbiota is one of the major defense mechanisms protecting the body against foreign bacteria, and disruption of this microbiota can harm the host. Endogenous microorganisms maintain the health of the host by preventing access of pathogenic bacteria and stimulating the immune response. Members of the indigenous microbiota may, however, cause local infections if the stability of the habitat is disrupted. Translocation of commensal oral bacteria to sites not normally accessible to them may also lead to infections at these non-oral sites. Some of these species, however, might not be involved in the etiology of the disease but merely be favored by the biological changes caused by true pathogens and host responses to them.

Although much progress has been made in the last 2 decades in the taxonomy of Gram-negative oral anaerobes, the high degree of heterogeneity among commensal bacteria, along with a high similarity with related species, can lead to problematic identification. Furthermore, clonal diversity within pioneering commensal species can be high, and several clones inhabit the oral cavity simultaneously. After initial colonization, commensal bacterial species tend to persist in the mouth for years.

The present thesis gives an overview of the identification of Gram-negative anaerobic bacteria of the oral cavity and considers some of their ecological and possibly virulent properties. Molecular methods were used for identification, taxonomic classification, investigating population dynamics, and tracing translocation of individual clones.

REVIEW OF THE LITERATURE

The oral cavity as a habitat for bacterial colonization

The ecological characteristics of the oral cavity make it unique in the body, although it should not be regarded as a uniform environment. The various surfaces of the oral cavity create diverse ecological niches, each with its own particular microbial population. Mucosal surfaces (tongue, cheeks, palate, and lips), teeth, and gingival crevices all form their own particular environment, and have their own specific microbial population, based on the physical and nutritional factors that apply to that particular site. A range of habitats that exist at each surface further increases this complexity of the mouth. Moreover, the properties of these environments are constantly changing. Daily changes include food consumption, hot and cold drinks, oral hygiene, and salivary flow. Thus the mouth may be considered a "feast or famine" environment. Not only does the ecology of the oral cavity change during the day, but also during the lifetime of the host, affecting the entire oral microbial community. In addition, other occasional events, such as scaling and polishing, dental restorations, and antibiotic therapy, influence the residential microbiota.

Bacterial populations in the oral cavity

Of the more than 10^{14} cells of which make the human body, only about 10% are mammalian. The remaining cells are the microorganisms that make the commensal microbiota of the host. The composition of this microbiota varies at distinct habitats, but is relatively consistent at each separate site within the individual. In healthy human subjects saliva contains roughly 10^{8} bacterial cells/ml and bacterial concentration of the gingival crevice exceeds 10^{11} bacteria/ml (Evaldson *et al.* 1982). In accordance with these high numbers of bacteria, between 500 and 1000 species of bacteria can be found in the oral cavity (Haffajee *et al.* 1999, Paster *et al.* 2001). These diverse bacteria can be divided into 2 categories based on their occurrence in the host; normal (resident) and transient (Loesche 1988). These normal species compose the commensal microbiota and are almost always found in high prevalence within the host. The transient species are less prevalent and in lower numbers. They may consist of transient bacteria, that are temporarily present in the oral cavity but disappear relatively quickly, but, the proportion of some of these species may occasionally increase, due to environmental circumstances, causing disease in a susceptible host.

Oral bacterial colonization in children

The composition of the oral microbiota varies with the age of the host. Age-related changes in the oral cavity include those due to teeth eruption, changes in diet, hormonal fluctuation, and salivary flow (Marcotte and Lavoie 1998). Infants are edentulous at birth, and their first teeth start to appear around the age of 6 months. By the age of 3 years, the primary dentition is usually complete and stays intact until at the age of 6 years when the permanent dentition starts to erupt.

Infants are susceptible to microbial colonization, as specific antibodies, such as secretory immunoglobulin A (sIgA), are present at relatively low levels in infancy (Könönen 2000). Additionally, some of the early colonizing bacteria have the ability specifically to cleave IgA1 *in vivo* (Frandsen *et al.* 1995b, Kilian *et al.* 1996), which aid these bacteria in evading the host immune response, and might even reduce the effect of the immune response on other bacteria lacking this capability. Although many bacteria gain access to the mouth, only certain species become established. Most of the bacteria found in the oral cavity at any particular time are only transient, while others find a suitable surface for attachment and growth. Colonization of the oral cavity is not a random event, but much rather a selective process with regard to the age at which infants are susceptible to colonization by different species of bacteria (Könönen 2000). Furthermore, colonization of each species alters the environment in the oral cavity, allowing other species to colonize. Thus, there is a steady increase in the diversity of the oral flora from birth to the climax community of the adult (Marsh and Martin 1992).

At birth, the oral cavity is usually void of microbes, but within few hours microorganisms from the environment, especially from the mother, become established. The pioneer species are members of “viridans” streptococci (Carlsson *et al.* 1970a, Carlsson *et al.* 1970b, Pearce *et al.* 1995, Rotimi and Duerden 1981). Initial anaerobic colonization of the oral cavity occurs within the first months of life (Könönen *et al.* 1992, Könönen *et al.* 1999c). In early childhood, children are exposed to bacterial clones similar to those present in their family members, and the species diversity increases in direct proportion with age. As they get older, contact with other persons increases and, children are exposed to a wider range of bacterial species and clones, for example in daycare.

Previously, anaerobes were considered to be absent from the mouth of infants, as it was assumed that they were dependent on the oxygen-depleted environment of the gingival crevice, i.e. the presence of teeth. Additionally, anaerobes were regarded as late

colonizers, not appearing until puberty (Bailit *et al.* 1964, Kelstrup 1966). Anaerobic bacteria are, however, frequently isolated from the oral cavity of edentulous infants (Könönen *et al.* 1992, Könönen 1999, Könönen *et al.* 1999c). The recent concept is that the strictly anaerobic bacteria coexist through coaggregation with oxygen-consuming bacteria, forming a biofilm, where various physical and chemical gradients, such as pH, oxygen concentration, and electric potential exists, thus offering a suitable environment for anaerobic bacteria (Kolenbrander 2000). The low redox potential within the papillary surface of the dorsum of the tongue might supply the necessary reservoir for the obligatory anaerobic bacteria in edentulous infants.

Attachment and coaggregation in oral bacterial colonization

Adhesion of bacteria to host surfaces is the initial event in the colonization of any environment (Gibbons 1984), and is essential for their growth and survival of bacteria in the mouth (Handley *et al.* 1999). If bacteria are unable to adhere to their surroundings, they will be rapidly removed by saliva flow. Colonization of the host tissues is accomplished by a variety of surface molecules, including fimbriae, adhesins and hemagglutinins, lipoteichoic acid, lipopolysaccharides, exopolysaccharides, outer membrane proteins, and outer membrane vesicles (Holt *et al.* 1999). Erythrocytes are widely used as a model for evaluating the specificity of bacterial adherence.

Coaggregation is the physical interaction between bacteria of different species. Coaggregations are not random among the oral bacteria, each species binds specifically to other bacteria. In dental plaque, certain bacteria often cluster together and if one member of a particular cluster is present in a sample, other members of that cluster are also most likely to be present as demonstrated using checkerboard DNA-DNA hybridization (Socransky *et al.* 1998). These observations have been supported by multiplex PCR studies (Yoshida *et al.* 2005). Some bacteria can adhere to few selected bacteria while *F. nucleatum*, which is the numerically dominating Gram-negative species in mature plaque, can adhere to all oral bacteria tested so far (Kolenbrander *et al.* 1989, Kolenbrander *et al.* 1999). Furthermore, if *F. nucleatum* is present in plaque, other anaerobes such as *P. nigrescens* and *P. melaninogenica* are usually also present (Socransky *et al.* 1998, Yoshida *et al.* 2005). *F. nucleatum* seems to play a central role in these mixed microbial communities, by offering protection to other obligately anaerobic bacteria such as *P. gingivalis* and *P. nigrescens* by forming mixed species aggregates with aerobic bacteria

(Bradshaw *et al.* 1996, Bradshaw *et al.* 1998). This protective role of *F. nucleatum* might be the cause for why colonization of *P. intermedia* seems to be dependent on *F. nucleatum* (Ali *et al.* 1994). Through these often complicated coaggregation complexes, biofilms are formed, where the bacteria have characteristics different from those they have in the planktonic form. In these large societies, bacteria often are inactive, and more tolerant to bactericidal factors, such as antiseptics, antimicrobials, redox potential, and oxygen (Costerton *et al.* 1999, Gilbert and Allison 1999).

Oral cultivable Gram-negative anaerobes

Although a considerable number of oral bacteria cannot be cultivated, conventional culture remains an essential method in examining the oral microbiota. Advances in culture techniques during the last 25-30 years have made obligately anaerobic bacteria a more feasible field of research. The use of anaerobic cabinets and gas exchange modules for anaerobic jars creates the anaerobic incubation environment within minutes. Anaerobic culture methods are sensitive to methodological errors and require a well-established and standardized approach. Anaerobes grow rather slowly, so their growth is not visible until at least 2 days after inoculation, and primary cultures take even longer to adapt to the incubation environment.

Prevotella and *Porphyromonas*

The heterogenic group of obligate anaerobes first described by Oliver and Wherry in 1921 as "*Bacterium melaninogenicum*" has undergone several taxonomical rearrangements through the years. Historically the oral *Bacteroides* were separated into those that produce pigment and those that do not. On one hand, bacteria that produced black or brown pigment on blood agar were identified as *Bacteroides melaninogenicus*, despite the reported phenotypic diversity within the group (Holdeman and Johnson 1982, Tanner *et al.* 1992) and on the other hand, the non-pigmenting strains were identified as *Bacteroides oralis*. The taxonomic importance of the pigment was, however, greatly overrated, as the pigmentation of colonies is largely dependent on medium composition. Indeed, some bacteria were transferred from one species to another when they were found to be pigment-producing (Holbrook and Duerden 1974). Currently, according to their ability to ferment glucose, bile-sensitive saccharolytic and moderately saccharolytic species were transferred to a new genus called *Prevotella* (Shah and Collins 1990) and asaccharolytic

species to a new genus called *Porphyromonas* (Shah and Collins 1988). Since then numerous new species have been described within these 2 genera (Avgustin *et al.* 1997, Collins *et al.* 1994, Downes *et al.* 2005, Fournier *et al.* 2001, Hirasawa and Takada 1994, Könönen *et al.* 1998a, Love *et al.* 1992, Love *et al.* 1994, Moore *et al.* 1994, Sakamoto *et al.* 2004, Sakamoto *et al.* 2005, Shah and Gharbia 1992), and additional species have been transferred there from other genera (Love *et al.* 1992, Love 1995, Shah *et al.* 1995a, Willems and Collins 1995a, Willems and Collins 1995b).

The species in the genus *Prevotella* form a heterogeneous group in the oral cavity. The indole-negative and lactose-fermenting *P. melaninogenica* group includes the phenotypically similar species *P. melaninogenica*, *P. loescheii*, and *P. denticola*, (Shah and Collins 1990). Similar to these are *P. oralis*, *P. veroralis*, *P. shahii*, *P. salivae*, *P. multiformis*, and *P. baroniae* (Downes *et al.* 2005, Sakamoto *et al.* 2004, Sakamoto *et al.* 2005, Shah and Collins 1990, Wu *et al.* 1992), although they have not been found to produce pigment. Members of the *P. melaninogenica* group are among the first anaerobic bacteria to colonize the mouths of infants (Könönen *et al.* 1992, Könönen *et al.* 1999c) and are regarded as a part of the commensal microbiota. The indole-positive and moderately saccharolytic *P. intermedia* group contains *P. intermedia*, *P. nigrescens*, and *P. pallens*. *P. intermedia* and *P. nigrescens* are phenotypically identical (Shah and Gharbia 1992) whereas *P. pallens* is lipase negative and only faintly pigmenting (Könönen *et al.* 1998a). *P. disiens* is similar but has not been found to produce pigment and is indole-negative. Bacteria of this group are frequently found in the oral cavity of both healthy individuals and patients with periodontal diseases.

The genus *Porphyromonas* includes the human oral species *P. gingivalis*, *P. endodontalis*, and *P. catoniae* and the non-oral species *P. asaccharolytica* and *P. uenonis* (Finegold *et al.* 2004, Shah and Collins 1988, Willems and Collins 1995b), and several non-human oral species (Collins *et al.* 1994, Fournier *et al.* 2001, Hirasawa and Takada 1994, Love *et al.* 1994). Recent 16S-23S rDNA internal transcribed spacer (ITS) sequencing shows the species separation of the *Porphyromonas* genus to be correct, although some heterogeneity is seen within some of the species (Conrads *et al.* 2005). *P. gingivalis* has been strongly linked to periodontal diseases (Consensus report 1996, Haffajee and Socransky 1994, Moore and Moore 1994, Slots 1999) and is rarely detected in healthy individuals (Griffen *et al.* 1998, Moore and Moore 1994). *P. endodontalis* is

particularly recovered from infected root canals and abscesses of odontogenic origin (Dahlén and Möller 1992).

Fusobacterium nucleatum

Difficulties with the taxonomy of the *Fusobacterium* genus are being resolved following the transfer of some species to other genera (Jalava and Eerola 1999), although studies still indicate genetic heterogeneity within the genus (Conrads *et al.* 2002). The genus *Fusobacterium* includes both human and animal species (Hofstad 1999, Jalava and Eerola 1999), but some are not genetically related to *Fusobacterium*, and further reclassification is still needed (Conrads *et al.* 2002, Hofstad 1999).

Among the *Fusobacterium* genus, *F. nucleatum* is the most significant species present in the oral cavity. *F. nucleatum* is a heterogeneous species (Thurnheer *et al.* 1999) and is currently divided into 5 subspecies (Dzink *et al.* 1990, Gharbia and Shah 1992), although the validity of these subspecies is disputed (Conrads *et al.* 2002, Morris *et al.* 1997, Olsen and Shah 2003, Paster *et al.* 2001). *F. nucleatum* is the numerically dominating Gram-negative species in mature plaque. Furthermore, it is one of the first anaerobic species to colonize the mouths of infants (Könönen *et al.* 1999c) and is one of the most commonly occurring species in the gingival crevice (Moore and Moore 1994). Because *F. nucleatum* coaggregates with all other oral bacteria tested so far, it is considered a key species in the building and development of complex biofilms of the oral cavity (Kolenbrander *et al.* 1999).

Identification of oral Gram-negative anaerobes

The purpose of identification is to match a strain with a previously recognized taxonomic group, using a small number of characters, which may be weighted. To be qualified as a good identification system, it must be reliable, convenient, rapid, include relatively few tests, be flexible, and economical in terms of materials and time. A wide variety of identification techniques exists and the majority of these require the organism to be isolated in pure culture, although culture-independent methods are becoming more established.

In order to be useful in demonstrating phylogeny, a DNA sequence must be present in all organisms, performing the same function, have sufficiently conserved nucleotide sequence, and be sufficiently large to deliver adequate phylogenetic

information. Molecules such as the ribosomal RNA genes, RNA polymerase, and elongation factor G have proved to provide valuable phylogenetic information (Tanner *et al.* 1994). The RNA of the small ribosomal subunit has been widely used for phylogenic research, and has been found to produce comparable phylogeny as the 23S rRNA molecule (Schleifer 1994). Currently (April 2004), the number of small subunit rRNA gene sequences in the Ribosomal Database Project (Maidak *et al.* 2001) is closing in on 140,000 sequences. According to 16S rRNA gene sequence analysis, the *Fusobacterium*, *Bacteroides*, *Prevotella*, and *Porphyromonas* species belong to a subgroup of the Bacteroidaceae family (Hofstad 1999, Logan 1994).

Phenotypic characteristics useful in identification

The taxonomy of the Gram-negative oral anaerobes was relatively cumbersome until in the 1990's when the *Bacteroides* genus was rearranged and the *Prevotella* and *Porphyromonas* genera were formed (Shah and Collins 1988, Shah and Collins 1989, Shah and Collins 1990). Later the phenotypically identical *P. intermedia* and *P. nigrescens* were separated (Shah and Gharbia 1992). Numerous identification methods for the *Prevotella* and *Porphyromonas* species have been described, both previously and after these taxonomic rearrangements. Most strains of *P. gingivalis*, *P. intermedia*/*nigrescens*, and the *P. melaninogenica* group produce pigment when they grow on blood agar. Although pigmentation is largely dependent on the culture media used (Tanner *et al.* 1992), pigment production remains a widely used distinguishing character in the early stages of identification. Some phenotypic characteristics used for identification of selected Gram-negative anaerobes are listed in Table 1.

Due to weak fermentation reactions, carbohydrate utilization tests are of limited value in routine identification of *F. nucleatum* (Morris *et al.* 1997). *F. nucleatum* can be separated from other common *Fusobacterium* species by their characteristic cell morphology: long, almost filamentous cell shape with pointed ends, but the separation of the *F. nucleatum* subspecies can only be achieved by molecular methods, such as multilocus enzyme electrophoresis (Lawson *et al.* 1989, Roques *et al.* 2000) or ITS sequencing (Conrads *et al.* 2002).

Table 1. Valuable characters for identification of some Gram-negative anaerobes covered in the present work (adapded from Jousimies-Somer *et al.* 2002).

	Susceptibility*							
Species/groups	Van	Kan	Col	Fluor*	Ind*	β-gal*	Try*	Lip*
P. melaninogenica group	R	R	V	red	-	+	-	-
P. intermedia/*nigrescens*	R	R		red	+	-	-	+
P. gingivalis	S	R	R	-	-	-	+	-
F. nucleatum	R	S	S	yellow	+			-

* Van = Vancomycin, Kan = Kanamycin, Col = Colistin, Fluor = direct UV fluorescence, Ind = indole, β-gal = β-galactosidase, Try = trypsin like activity, Lip = lipase.

Molecular methods for identification

Phylogenetic analysis of a number of *P. intermedia* and *P. nigrescens* strains indicates that variations in 16S rRNA gene sequences within the species are relatively low but the difference between these species is more than 7% (Kuhnert *et al.* 2002). This difference makes the 16S rRNA gene a good candidate for species-specific PCR separation as applied by numerous researchers, both qualitatively on colonies from primary cultures (Conrads *et al.* 1997, Mättö *et al.* 1996a, Okamoto *et al.* 1999, Premaraj *et al.* 1999, Shah *et al.* 1995b, Slots *et al.* 1995); and directly from clinical samples (Ashimoto *et al.* 1996, Riggio *et al.* 1998, Stubbs *et al.* 1999); or quantitatively from clinical samples (Doungudomdacha *et al.* 2000, Gmür and Thurnheer 2002, Kuboniwa *et al.* 2004, Martin *et al.* 2002). Furthermore, the 16S rRNA gene has been the target for simultaneous detection of different species by multiplex PCR, including *P. intermedia* (Conrads *et al.* 1999, García *et al.* 1998) and *P. nigrescens* (Yoshida *et al.* 2005). These methods have proved to be fast and accurate for the separation of *P. intermedia* and *P. nigrescens*. Various other molecular methods have been used to separate *P. nigrescens* from *P. intermedia*, such as multilocus enzyme electrophoreses (Frandsen *et al.* 1995a, van Steenbergen *et al.* 1997), restriction enzyme analysis of total DNA (Teanpaisan *et al.* 1996), monoclonal antibodies (Devine *et al.* 1994), SDS-PAGE protein electrophoresis (Frandsen *et al.* 1995a), 16S rDNA PCR-restriction fragment length polymorphism (PCR-RFLP) (Milsom *et al.* 1996), and ribotyping (Mättö *et al.* 1996b, Teanpaisan *et al.* 1996).

Approximately 50% of the oral microbiota remain unrecognized by conventional culture methods (Wade 1999). However, a snapshot of the entire bacterial population can be achieved by direct amplification of the 16S rRNA genes or molecules by PCR. After singularizing the amplicons by cloning, they can either be re-amplified for use in restriction fragment length analysis or directly sequenced and compared to existing sequences in the GeneBank (http://www.ncbi.nlm.nih.gov/) or RDP-II (http://rdp.cme.msu.edu/) databases. These advanced culture-independent methods are used for investigating the total bacterial population of various habitats and infections in the oral cavity. These methods have identified numerous uncultivable and previously unknown phylotypes in endodontic infections (Munson *et al.* 2002), dentoalveolar abscess (Dymock *et al.* 1996, Wade *et al.* 1997), subgingival plaque (Choi *et al.* 1994, Paster *et al.* 2001, Spratt *et al.* 1999), caries lesions (Becker *et al.* 2002, Munson *et al.* 2004), and carious dentin (Nadkarni *et al.* 2004). In a detailed study (Paster *et al.* 2001), with 2522 clones from subgingival plaque from subjects with and without periodontal diseases, about 60% of the clones fell into 132 known species. However, the rest of the clones represented novel phylotypes, of which many were found in multiple subjects. Notably, proportions of certain bacteria can differ considerably between cultivation and direct amplification (Dymock *et al.* 1996, Munson *et al.* 2004), indicating selectivity in the culture methods.

Population structure and dynamics within oral commensals

Various molecular methods, among them RFLP of total DNA, pulsed-field gel electrophoresis (PFGE) of low frequency restriction of total DNA, ribotyping, serotyping, DNA sequencing, DNA probing, and DNA amplification procedures, such as arbitrary primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD), are nessecary to identify clones of numerous bacterial species (Vandamme *et al.* 1996). Clonal typing is needed for investigating the population dynamics within bacterial populations, clonal diversity of separate species, translocation from one site to another intra-individually, and transmission of bacteria from one individual to another.

Molecular methods are necessary for investigating the population structure and dynamics of oral bacterial species. Generally, the intra-individual clonal diversity of oral early-colonizing commensals seems to be rather high; several clones of facultative *Streptococcus mitis* (Hohwy *et al.* 2001), *Streptococcus sanguis* (Pan *et al.* 2001), and *Eikenella corrodens* (Chen and Ashimoto 1996, Fujise *et al.* 2004), as well as strictly

anaerobic *P. melaninogenica* (Könönen *et al.* 1994a, Könönen *et al.* 1994c) and *F. nucleatum* (George *et al.* 1997, Suchett-Kaye *et al.* 1998, Thurnheer *et al.* 1999), simultaneously reside in each individual. Furthermore, *Streptococcus mutans*, the main species associated with dental caries, demonstrates high clonal variation intra-individually (Grönroos and Alaluusua 2000, Klein 2000, Redmo Emanuelsson *et al.* 2003). In general, clonal variation seems to be relatively low among oral pathogens, and usually only one or 2 clones of *P. gingivalis* and *P. intermedia* inhabit each individual at a given time (Saarela *et al.* 1993a, Saarela *et al.* 1993b, Teanpaisan *et al.* 1996, van Steenbergen *et al.* 1993b), even though the clonal heterogeneity of these bacteria between individuals is high (Loos *et al.* 1990, Ménard and Mouton 1995). Similarly, only one or 2 clones of *Actinobacillus actinomycetemcomitans* usually reside in each individual (Alaluusua *et al.* 1993). On the other hand, the opposite has been found in beagle dogs where 4-8 genotypes of *P. gingivalis* were found in a single individual dog (Madianos *et al.* 1994). It still remains to be seen whether this difference in intra-individual clonal heterogeneity between commensals and pathogenic bacteria is true or an artifact of the research methods used.

The population dynamics of oral commensal species has previously been studied among *S. mitis* isolates from 2 infants and their parents. In infants, this pioneering species showed a high clonal variation and rapid turnover of clones. The situation was different in their parents, where clonal persistence was common (Hohwy *et al.* 2001). The clonal turnover of another early-colonizing species, *P. melaninogenica*, is also quite high in young children. In a study containing 9 children, only one dentate child harbored the same *P. melaninogenica* ribotype as it had as edentulous infant (Könönen *et al.* 1994a). In a study containing 11 mother-child pairs, only one child harbored the same ribotype in infant and child period. In 5 of the mothers, however, one or 2 *P. melaninogenica* ribotypes persisted (Könönen *et al.* 1994c). Clonal stability of the commensal species *E. corrodens* (Fujise *et al.* 2004) and *F. nucleatum* (Suchett-Kaye *et al.* 1998) in adults seems, however, relatively low.

Transmission and translocation of oral bacteria

Numerous researchers have shown transmission of oral bacteria between individuals. Infants and young children require their oral microbes from their close contacts, especially from their mothers (Caufield and Walker 1989, de Soet *et al.* 1998, Klein 2000, Köhler and Bratthall 1978, Könönen *et al.* 1994c, Könönen *et al.* 2000) but also from their fathers

(Redmo Emanuelsson and Wang 1998), and from other individuals with frequent contacts, such as siblings and other children at daycare (Mattos-Graner *et al.* 2001). Furthermore, identical clones of some periodontal pathogens and mutans streptococci have been demonstrated to inhabit spouses (Redmo Emanuelsson and Wang 1998, Saarela *et al.* 1993b, Suchett-Kaye *et al.* 1999, van Steenbergen *et al.* 1993b, van Steenbergen *et al.* 1997) and the same bacterial clones can be frequently found in all members of a family (Asikainen *et al.* 1996, Redmo Emanuelsson and Wang 1998, Suchett-Kaye *et al.* 1999).

Translocation of bacteria from one site to another in the oral cavity can occur and identical clones of bacteria are frequently found at different sites in the mouth (van Steenbergen *et al.* 1993b, van Steenbergen *et al.* 1997). Saliva contains the same bacterial clones as are found in subgingival plaque (Mättö *et al.* 1996a), and identical clones have been isolated from subgingival plaque and infected root canals (Gonçalves *et al.* 1999). Translocation of periodontal pathogens from one site to another can endanger the outcome of periodontal treatment (Quirynen *et al.* 2001) and, in fact, the re-emergence of common periodontal pathogens after periodontal treatment seems to be mainly from the indigenous microbiota, although transmission from spouses occur (von Troil-Lindén *et al.* 1996).

Bacterial translocation within the oral cavity as well as from the mouth to non-oral sites is most likely mediated through saliva. By swallowing, oral microorganisms are easily transferred to the pharynx (Hohwy and Kilian 1995) and the gastrointestinal tract (Hossain *et al.* 2003). Oral bacteria can also be translocated to the respiratory tract. Supine position, common among infants and debilitated elderly people makes them especially prone to bacterial translocation by aspiration of oral secretions (Loesche and Lopatin 1998, Scannapieco 1999). Indeed, many oral bacteria have been found in lung infections, both in abscesses (Brook and Frazier 1993) and pneumonia (Shinzato and Saito 1994). These include many anaerobes such as *Porphyromonas*, *Prevotella*, and *Fusobacterium* species (Brook and Frazier 1993, Loesche and Lopatin 1998, Shinzato and Saito 1994). Furthermore, the anatomical proximity of the subgingival microflora to the bloodstream can facilitate systemic spread of bacteria. Microorganisms gaining entrance to the bloodstream are usually eliminated by the host defense system within minutes, but in patients with defective heart valves or vascular diseases, bacteremia can be a potential danger, leading to infective endocarditis (Debelian *et al.* 1994, Loesche 1997). Indeed, in patients with infective endocarditis, the same clones of viridans streptococci are found in blood cultures and the oral cavity (Fiehn *et al.* 1995). Other forms of systemic diseases

such as brain abscesses, hematological infections, and implant infections have also been related to oral microorganisms (Debelian *et al.* 1994, Loesche 1997).

Virulence and virulence factors

A pathogen is a microbe that is capable of causing host damage, either from direct microbial actions or through the host immune response. Virulence is defined as the relative capacity of a microbe to cause damage in a host and virulence factors are those molecules, or components of a microbe, that have a damaging effect on host cells (Casadevall and Pirofski 1999, Holt *et al.* 1999). However, the strict definition of a pathogen, as above, excludes those microorganisms that cause disease only in the presence of other pathogens. Definitions of virulence factors are also problematic for the commensal microbiota, as it is difficult to distinguish virulence determinants from common traits (Casadevall and Pirofski 1999). In order to cause infection a microbe must have the ability to be transmitted to a susceptible host, enter the host, find a unique ecological niche, avoid host defenses, compete with the resident microbiota, replicate, and express specialized pathogenic traits (Fives-Taylor *et al.* 1999, Slots 1999). For the present purpose, all factors that attribute to the colonization, proliferation, and pathogenicity of microbes will be considered virulence factors.

Adherence of the bacterium to host cells is the initial step in colonization and pathogenicity. Access to the mouth is relatively direct, but less so for other sites in the body. Bacteria must penetrate barrier layers, such as surface slime layers on the mucous membranes, before they can become associated with the host cells and the cell or tissue can be invaded. After the initial colonization, the bacterium must compete with the resident microbiota often by producing numerous antibacterial molecules, such as bacteriocins, organic acids, alcohols, inorganic bases, ammonia, and other metabolic end products. Limiting nutrients are obtained by scavenging proteins, where iron-binding proteins are particularly important. Bacteria also have to shield themselves from the host defense system. By producing exopolysaccharide capsules bacteria can imitate host tissue and evade phagocytes (Holt *et al.* 1999). A variety of enzymes, which specifically cleave host immunoglobulins (Kilian 1981) or enzymes such as cysteine proteinases, which destroy host tissues are also produced (Holt *et al.* 1999). Furthermore, although not a direct virulence factor, many bacteria carry genes for antibiotic resistance, and in that way

can avoid the specific treatments of the diseases (Andersson 2003, Handal and Olsen 2000).

Virulence factors of Gram-negative anaerobes

Many *Prevotella* species possess virulence factors, for example, fimbria (Leung *et al.* 1996, Weiss *et al.* 1989), hemolysins (Allison and Hillman 1997, Beem *et al.* 1998, Beem *et al.* 1999, Takada *et al.* 2003), adhesins (Leung *et al.* 1989, Manch-Citron *et al.* 1992), and hemagglutinins (Leung *et al.* 1999, Okamoto *et al.* 1999). These bacteria commonly produce immunoglobulin-degrading enzymes (Frandsen *et al.* 1995b, Jansen *et al.* 1995, Kilian 1981), and some produce tissue-degrading enzymes (Slots and Genco 1984). Additionally, bacteria of the genus *Prevotella* are often resistant to antibiotics, such as tetracycline, erythromycin, and β-lactam antibiotics (Arzese *et al.* 2000, Dubreuil *et al.* 2003, Könönen *et al.* 1995, Könönen *et al.* 1997, Lacroix and Walker 1996, Mättö *et al.* 1999, Nyfors *et al.* 1999, Olsvik *et al.* 1996, Walker and Bueno 1997).

F. nucleatum is capable of agglutinating and lysing erythrocytes (Gaetti-Jardim and Avila-Campos 1999), binding to leukocytes (Ozaki *et al.* 1990), adhering to and invading epithelial cells (Han *et al.* 2000), producing serine protease capable of degrading extracellular matrix proteins (Bachrach *et al.* 2004), and activating leukocytes (Sheikhi *et al.* 2000) and lymphocytes (Tuttle *et al.* 1992). Furthermore the heat shock proteins of *F. nucleatum* can be found in the outer membrane, making them probable virulence factors (Skår *et al.* 2003). No significant differences have been found between the *F. nucleatum* subspecies considering virulence factors (Roques *et al.* 2000), although some clonal variation exists in ability to bind to lymphocytes, erythrocytes, and fibroblasts (Ozaki *et al.* 1990, Tuttle *et al.* 1992). Because of its multigeneric coaggregation ability, *F. nucleatum* can attach other bacterial species to a growing biofilm (Kolenbrander *et al.* 1999). Moreover, combinations of bacteria may synergistically produce more damage to host tissues than a single species (Bolstad *et al.* 1996). *F. nucleatum* has been shown to increase virulence of mixed infections in animal models (Brook and Walker 1986), modulate the host response of mice when infected with *P. gingivalis* (Choi *et al.* 2001), and support the growth of *P. gingivalis* and *P. nigrescens* when the culture is exposed to air (Bradshaw *et al.* 1998, Diaz *et al.* 2002). Furthermore, tetracycline resistance has been found in *F. nucleatum* (Olsvik and Tenover 1993) and these bacteria are frequent producers of β-lactamase (Könönen *et al.* 1999b, Nyfors *et al.* 2003).

P. gingivalis possesses various virulence factors, including fimbriae (Du *et al.* 1997, Yoshimura *et al.* 1984), trypsin like proteinase (Slots and Genco 1984), cysteine protease that also acts as a hemagglutinin (Lépine and Progulske-Fox 1996, Nishikata *et al.* 1989), adhesins (Agnani *et al.* 2000), hemolytic toxins (Chu *et al.* 1991, Deshpande and Khan 1999, Hoshi *et al.* 1993, Karunakaran and Holt 1993, Shah and Gharbia 1989, Shah *et al.* 1992), and more as has been reviewed by Holt *et al.* (1999). This species is also able to adhere to and invade oral epithelial cells (Sandros *et al.* 1993), and multiply and persist within them (Madianos *et al.* 1996).

Oral bacteria and diseases

The indigenous microbiota is one of the major defense mechanisms that protect the human or animal body, by preventing access of pathogenic bacteria and stimulating the host immune response, and the disruption of this microbiota may damage the host (Casadevall and Pirofski 2000, Tancrède 1992, Wilson 1974). However, some of the resident species have the potency to cause local or systemic disease, if disturbances occur that upset the stability of the habitat. These disturbances can be exogenous, such as antibiotic treatment, or they can be derived from endogenous changes, such as weakened host defenses. The unexpected presence of bacteria at sites not normally accessible to them may lead to infections at non-oral sites.

Local infections in the oral cavity are of 2 main types; dental caries and periodontal diseases. These infections are the result of complex interactions between the resident microbiota and the host. Dental caries is the decalcification of enamel by acid produced primarily by bacteria of supragingival dental plaque. This is highly dependent on the carbohydrate consumption of the host. Periodontal diseases are a group of disorders affecting the supporting tissues of the teeth, through an interplay between subgingival microorganisms and the host immune system, which can eventually lead to loss of teeth.

By using checkerboard DNA-DNA hybridization, Socransky *et al.* (1998) found certain bacteria often clustered together in dental plaque. If one member of a particular cluster was present in a sample, other members of that cluster were also most likely to be present. One of these clusters contained the highly suspected periodontal pathogens *P. gingivalis*, *Tannerella forsythensis* [*Bacteroides forsythus*], and *Treponema denticola*, which were found more frequently in deep periodontal pockets than in healthy sites. Bacteria of the genera *Fusobacterium*, *Prevotella*, *Campylobacter*, *Peptostreptococcus*,

and others, which are moderately connected with diseases, comprised another cluster. These 2 clusters were associated with periodontal diseases and furthermore, they were more associated with each other than with other clusters, indicating that these bacteria prefer similar living environment, or that they may be a causative agent for periodontal diseases.

Gram-negative anaerobes and diseases

Gram-negative anaerobic bacteria are frequent findings in periodontal diseases and other oral and non-oral infections.

F. nucleatum is one of the most common species in both supragingival and subgingival plaque in both healthy individuals and patients with periodontal disease (Könönen *et al.* 1994b, Moore and Moore 1994, Ximenez-Fyvie *et al.* 2000). It has been associated with infections in the head and neck area, particularly with early stages of periodontal diseases in adults, since its prevalence (Darby and Curtis 2001) and proportion (Moore *et al.* 1985) is significantly higher in diseased sites than in healthy sites. Furthermore, it is frequently found in endodontic infections (Bolstad *et al.* 1996, Dahlén and Möller 1992, Moraes *et al.* 2002). There is high heterogeneity within the species and, although limited data exist, it is possible that each subspecies may have different primary habitats and may be involved in different types of infections and specific clinical stages of disease (Bolstad *et al.* 1996, Finegold and Jousimies-Somer 1997, Gharbia *et al.* 1990). Moreover, *F. nucleatum* is commonly found in various types of clinical infections of other body sites (Bolstad *et al.* 1996, Brook and Walker 1986, Chryssagi *et al.* 2001, Moore and Moore 1994). In children, *F. nucleatum* can be found in abscesses, respiratory tract infections (Brook 1994), in the nasopharynx of children during acute otitis media (AOM) episodes (Könönen *et al.* 1999a), and from middle ear effusion from children with chronic otitis media (Brook *et al.* 2000, Külekci *et al.* 2001).

The *P. melaninogenica* group includes the least virulent species of the *Prevotella* (Kamma *et al.* 2000, Slots and Genco 1984). These species are frequent findings in healthy individuals, and are usually not associated with diseases (Könönen 1993, Könönen *et al.* 1994a, Könönen *et al.* 1994b, Ximenez-Fyvie *et al.* 2000), although the prevalence (Wu *et al.* 1992) and the proportion (Moore *et al.* 1985) of some of the bacteria of the *P. melaninogenica* group increases with the severity of periodontal disease. These bacteria have been isolated from children with otitis media, both from the nasopharynx (Könönen

et al. 1999a) and from the middle ear of children with middle ear effusion (Brook 1987) and chronic otitis media (Brook 1996, Brook *et al.* 2000). They are, furthermore, associated with certain clinical symptoms in endodontic infections (Drucker *et al.* 1997).

Moderate associations exist between *P. intermedia*/*nigrescens* and periodontal diseases (Haffajee *et al.* 1999). The *P. intermedia* "sensu lato" was previously linked to periodontal diseases, however, the validity of earlier investigations remains uncertain since these 2 separate species may have difference in their virulence properties. Since the separation of *P. nigrescens* from *P. intermedia* (Shah and Gharbia 1992), researchers have described an association between *P. intermedia* and periodontal diseases, whereas *P. nigrescens* seems to be mainly connected with healthy gingiva (Dahlén *et al.* 1990, Gharbia *et al.* 1994, Mättö *et al.* 1996b, Teanpaisan *et al.* 1995, Ximenez-Fyvie *et al.* 2000). *P. nigrescens* is, however, more frequently found in infected root canals and non-oral abscesses than *P. intermedia* (Finegold and Jousimies-Somer 1997, Gharbia *et al.* 1994). *P. intermedia*/*nigrescens* is also a common finding in children with chronic otitis media (Brook 1995). No significant difference in binding characteristics or pathogenicity of the 2 species has, however, been observed (Dahlén *et al.* 1996, Hafström and Dahlén 1997).

Of the bacteria included in the current investigations, the strongest association is between periodontal diseases and *P. gingivalis*. *P. gingivalis* is rarely found in healthy individuals but is found in high prevalence in periodontitis (Darby and Curtis 2001, Finegold and Jousimies-Somer 1997, Griffen *et al.* 1998, Haffajee and Socransky 1994, Haffajee *et al.* 1999, Moore and Moore 1994, Ximenez-Fyvie *et al.* 2000, Zambon 1996). *P. gingivalis* is also commonly found in necrotizing ulcerative gingivitis, infected root canals, peritonsillar abscesses, and abscesses of periodontal and endodontic origin (Dahlén and Möller 1992, Gmür *et al.* 2004, Mättö *et al.* 1997), but only occasionally from infections outside the oral cavity (Mättö *et al.* 1997).

There has been an increasing awareness of possible associations between periodontal disease and systemic disorders. It has been speculated that Gram-negative anaerobic bacteria in periodontitis are associated directly, or through their stimulation of an immune response, with systemic disorders, especially with cardiovascular diseases (Beck and Offenbacher 2001) and preterm birth (Jeffcoat *et al.* 2001, Offenbacher *et al.* 1996). These associations are disputed, especially with respect to preterm birth (Davenport *et al.* 2002, Holbrook *et al.* 2004). The variation between these reports might

be due to the fact they dealt with different populations and the associations might be racially or lifestyle linked, indicating that the genetic background of the host affects the reaction to the colonizing bacteria.

WORKING HYPOTHESES AND AIMS OF THE STUDY

Working hypotheses

1. Hemagglutinating bacterial strains resembling *P. melaninogenica* form a separate species inside the *Prevotella* genus and bind to erythrocytes with similar strength and by mechanism, similar to the major periodontal pathogen, *Porphyromonas gingivalis*.

2. Clonal diversity and frequent turnover of clones are common within early colonizing oral anaerobic bacterial populations.

3. Anaerobic bacteria that transiently colonize the nasopharynx during respiratory infection are of oral origin.

Aims

The general aim of this study was to develop an identification scheme and clonal typing methods for some oral commensal and pathogenic Gram-negative anaerobic bacteria. The specific aims were:

1) to develop a simple identification scheme for selected oral Gram-negative anaerobes that could be applied to clinical research.

2) to investigate hemagglutinating isolates resembling *P. melaninogenica* by determining whether these isolates form a separate species and clarifying hemagglutination properties of these isolates.

3) to develop an arbitrarily-primed PCR (AP-PCR) method for genotyping *F. nucleatum*, with the view to examine the population structure and dynamics of this common anaerobic species.

4) to demonstrate the oral origin of *F. nucleatum* isolates from the nasopharynx of infants with acute otitis media experience.

MATERIAL AND METHODS

Table 2 summarizes the subjects, bacterial isolates, and methods and Table 3 summarizes the reference strains included in studies I-V and in unpublished data.

Subjects, sample collection, and primary cultures

Seventy-six adult subjects attending the Dental School in Reykjavík, Iceland, for a routine dental examination were enrolled in the study on pigmented Gram-negative anaerobes. Following clinical and radiographic examination and probing of pocket depths, the periodontal status was recorded. Pooled paperpoint samples were taken from 2-4 subgingival sites of 49 healthy subjects with no pockets >3mm and from 27 patients with periodontitis. The samples were transported in a VMG II medium (Dahlén *et al.* 1993), and processed within one hour in the laboratory. Brown and black colonies of Gram-negative anaerobes were subcultured and, once pure, harvested and kept frozen at -80°C until further use (**I, II, III**).

Sixteen infants positive for *F. nucleatum* originated from a satellite subpopulation of the Finnish Otitis Media (FinOM) cohort study, where 50 healthy, at baseline 2-month-old Caucasian infants were recruited to a prospective, longitudinal study on the development of the microflora in the upper respiratory tract (Könönen *et al.* 2002). The infants had been followed in a study clinic at scheduled healthy visits up to 24 months of age and, in addition, between the visits if an infant became sick. Their infections had been diagnosed and treated in the same clinic as described in detail by Syrjänen *et al*. (2001). Unstimulated saliva from the buccal sulcus area of the mouth and nasopharyngeal swab (NP) samples had been collected at scheduled healthy visits at the age of 2 (+/- 2 weeks), 6 (+/- 2 weeks), 12 (+/- 2 weeks), 18 (+/- 4 weeks), and 24 (+/- 4 weeks) months and nasopharyngeal aspirate (NPA) samples at every visit related to AOM. All samples had been cultured within 24 hours after collection on several media, including neomycin-vancomycin agar selective for fusobacteria, and the isolates identified using established biochemical methods as described previously (Könönen *et al.* 1999c, Könönen *et al.* 2003) (**IV, V**).

Clinical isolates and reference strains

A total of 246 clinical isolates of dark-pigmented, Gram-negative, anaerobic rods, and 43 well-characterized reference strains of *Prevotella*, *Porphyromonas*, and *Fusobacterium*

Table 2. Origin of clinical isolates and methods used in the studies.

Subjects	Isolates	Methods
Study I		
76 adults (49 periodontally healthy, 27 with periodontitis)	246 isolates of dark-pigmented Gram-negative anaerobes isolated from subgingival sites	Phenotypic tests API 20A and rapid ID 32A test kits 16S rDNA based PCR
Study II		
18 adults positive for *P. melaninogenica* (a subset of the 76 subjects in Study I)	9 *P. melaninogenica* isolates positive for HA* on microscope slide 12 *P. melaninogenica* isolates negative for HA on microscope slide	16S rDNA PCR-RFLP Negative staining electron microscopy
Study III		
12 adults positive for *P. melaninogenica* (a subset of the 76 subjects in Study I)	16 *P. melaninogenica* isolates	Microscope slide HA assay Microtiter plate HA assay Inhibition of HA in microtiter plate assay
Study IV		
12 infants positive for *F. nucleatum* on at least 3 subsequent samplings	546 *F. nucleatum* isolates from saliva	AP-PCR genotyping

Study V		
8 infants positive for nasopharyngeal *F. nucleatum*	11 *F. nucleatum* isolates from the nasopharynx 161 *F. nucleatum* isolates from saliva	AP-PCR genotyping
Unpublished		
5 adults (2 periodontally healthy, 3 with periodontitis)	63 *P. melaninogenica* isolates	Microscope slide HA assay
28 pregnant women	1109 *P. intermedia*/*nigrescens* isolates	16S rDNA based PCR

* HA = hemagglutination

(Table 3) were used to develop a simple identification scheme. In addition, phenol-extracted DNA was available from further 16 strains of *Prevotella* and *Porphyromonas* for PCR (**I**). As a subset of the previous material, 38 clinical isolates resembling *P. melaninogenica* and 22 well-characterized reference strains of various *Prevotella* spp. (Table 3) were used for taxonomical classification (**II**). Sixteen clinical *P. melaninogenica* isolates and 7 reference strains representing *P. melaninogenica*, *P. intermedia*, and *P. gingivalis* were included in investigating strength of hemagglutination. For further tests on hemagglutinating properties of *P. melaninogenica*, 2 clinical isolates (G9 and G107) were selected as hemagglutinating representatives of the 2 clusters found in Study II, and one isolate (G11) was selected as a non-hemagglutinating representative (**III**).

Altogether 546 salivary *F. nucleatum* isolates from 12 infants (mean 45.5 isolates/subject) were used to investigate population structure and kinetics among developing anaerobic commensals of the mouth (**IV**). Eleven *F. nucleatum* isolates from the nasopharynx of 8 infants and 161 isolates from saliva (mean 20.1 salivary isolates/subject) of the same infants were available for investigating the genetic similarity or dissimilarity of nasopharyngeal and salivary *F. nucleatum* (**V**). The type strains of each human *F. nucleatum* subspecies (Table 3) were used as reference strains (**IV, V**).

Development of identifications schemes (I)

Three different identification methods were compared in order to develop a simple and rapid identification scheme for pigmented Gram-negative anaerobes.

1) Screening with 5 phenotypic tests: Gram stain reaction and cell morphology; direct fluorescence in UV light; detection of β-galactosidase activity (as an indicator of lactose fermentation) using 4-methylumbelliferyl-β-D-galactoside (MUG; Sigma-Aldrich, St. Louis, MO, USA); detection of trypsin-like activity using carbobenzoxy-L-arginin-7-amino-4-metylcoumarin amide-HCl (CAAM; Fluka Chemie AG, Buchs, Switzerland); and agglutination of human erythrocytes on microscope slides (Slots and Genco 1979).

2) Use of 2 commercial identification kits, the API 20A and rapid ID 32A (BioMérieux, Marcy-l'Étoile, France).

3) A 16S rDNA-based PCR method using primers specific for *P. melaninogenica*, *P. intermedia*, *P. nigrescens*, and *P. gingivalis* (Table 4).

Table 3. A list of species and number of strains used as references.

Species	Total number of strains	Study
P. bivia	1	**I, II**
P. buccae	1	**I**
P. buccalis	1	**I**
P. corporis	1	**I, II**
P. denticola	1	**I, II**
P. disiens	1	**I, II**
P. intermedia	12	**I, II, III, unpublished**
P. loescheii	1	**I, II**
P. melaninogenica	10	**I, II, III, unpublished**
P. nigrescens	14	**I, II, unpublished**
P. oralis	2	**I, II**
P. oris	1	**I, II**
P. oulora	1	**I, II**
P. pallens	1	**I**
P. tannerae	1	**I**
P. veroralis	1	**I, II**
P. asaccharolytica	1	**I**
P. gingivalis	10	**I, III**
F. nucleatum subsp. *fusiforme*	1	**IV, V**
F. nucleatum subsp. *nucleatum*	1	**IV, V**
F. nucleatum subsp. *polymorphum*	1	**I, IV, V**
F. nucleatum subsp. *vincentii*	1	**IV, V**

Hemagglutination assays (II, III, unpublished)

The hemagglutination of erythrocytes from human, sheep, rabbit, rat, guinea-pig, and horse was tested. For the microscope slide assay, bacterial growth was suspended in erythrocyte suspension on a microscope slide and hemagglutination visualized in microscope at 10-fold magnification. For microtiter plate assay, bacterial growth was suspended in PBS to optical density (OD) 1.000 at 550 nm concentration, 2-fold dilutions

of bacterial strains were made in microtiter plates with V-shaped bottoms, equal volume of 2% erythrocyte suspension added to each well, and incubated at 4°C for 4 h.

Inhibition of hemagglutination by microtiter plate assay (III)

Inhibition of hemagglutination of isolates G9, G107, and G11 by 6 sugars by adding each sugar to the bacterial/erythrocyte suspension in 1% and 2% (wt/vol) final concentration. The effect of adding proteinase inhibitors was also tested. Furthermore, the effect of heating the bacteria, treating bacteria with proteolytic enzymes, shaking of bacteria, and neuramidinase and proteolytic enzyme treatment of the erythrocytes on hemagglutination was tested.

DNA isolation for PCR (I, II, IV, V, unpublished)

One or 2 large colonies of a young culture were harvested from an agar plate using a sterile loop, suspended in 500 µl of 5% Chelex 100 (Bio-Rad Laboratories, Hercules, CA, USA) and boiled for 10 min. The suspension was then shaken lightly on a Vortex mixer, and centrifuged and the supernatant used for PCR amplification. Furthermore, phenol-extraction of DNA was used on 16 additional reference strains in Study I.

Oligonucleotide primers and PCR methods (I, II, IV, V, unpublished)

The primers used for PCR are listed in Table 4.

PCR for species identification was performed in 40 µl volumes in a 96-well microtiter plate (Techne Ltd, Cambridge, UK) in a DNA thermal cycler (Techne Ltd). The amplification for *P. intermedia*, *P. melaninogenica*, and *P. gingivalis* primer pairs was at primer annealing temperature of 60°C for 1 min, but with the *P. nigrescens* primer pair at 66°C. Amplified material were stored at 4°C until viewed by 1.5% agarose (MedProbe, Oslo, Norway) electrophoresis, staining with ethidium bromide, and photographing under UV light. A HaeIII-digested ΦX 174 (Gibco BRL, Paisley, UK) served as a molecular weight marker (**I, II**).

PCR-restriction fragment length polymorphism (PCR-RFLP) of the 16S rDNA was performed for taxonomical classification. The 16S rDNA was amplified using the slightly modified primers fD1 and rD1 of Weisburg *et al.* (1991). The amplified 16S rDNA was digested with *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, and *Taq*I endonucleases (Gibco BRL) in 5 separate digestions. The digested amplicons were stored at -20°C until separated by

Table 4. Oligonucleotide primers used.

Reaction	Specificity	Primer name	Oligonucleotide sequence 5'-3'	Study
Identification PCR	*P. melaninogenica*	SJ 86	CTACATTTCACAACACACTTAATCT	**I, II**
		SJ 87	AAACGGCATTGAGTGCTTGCACTCT	**I, II**
	P. nigrescens	PN-1-kort	TTGAGTACACGCAGCGCAGGCG	**I, unpublished**
		PN-3	CCCGATGGCAACTGGGAAAGG	**I, unpublished**
	P. intermedia	PI-3	CCCGATGTTGTCCACATATGG	**I, unpublished**
		PI-4	GCATACGTTGCGTGCACTCAAG	**I, unpublished**
	P. gingivalis	SJ 96	ACTGTTAGCAACTACCGATGT	**I**
		SJ 97	AGGCAGCCTGCCATACTGCG	**I**
	Positive control	KO-1	CCCGGGAACGTATTCAACG	**I, II, unpublished**
	16S rDNA	KO-2-st	GATTAGATACCCTGGTAGTCC	**I, II, unpublished**
PCR-RFLP	16S rDNA	fD1	AGAGTTTGATCCTGGCTCAG	**II**
		rD1	AAGGAGGTGATCCAGCC	**II**
AP-PCR	non-specific	C1	GATGAGTTCGTGTCCGTACAACTGG	**IV, V**
	non-specific	C2	GGTTATCGAAATCAGCCACAGCGCC	**IV, V**
	non-specific	D8635	GAGCGGCCAAAGGGAGCAGAC	**IV, V**
	non-specific	D11344	AGTGAATTCGCGGTGAGATGCCA	**IV, V**

2% agarose (MedProbe) electrophoreses, stained with ethidium bromide, and photographed under UV light. A *Hae*III digested ϕX 174 (Gibco BRL) served as a molecular weight marker. Photographs were scanned, digitized using a PDI scanner, and a PDI-user program (PDI, Huntington Station, New York, USA) (**II**).

AP-PCR was performed for clonal typing in a 25 µl volume in a 500 µl Ready-To-Go-PCR™ tube (Amersham Biosciences), using one of the 4 primers in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). A negative control (without DNA) was included in each AP-PCR run. Amplification was performed using a slightly modified method of George *et al*. (1997). Amplified products were kept at 4°C until separated by 1.5% SeaKem agarose (FMC, Bioproducts, Rockland, ME, USA) electrophoresis, stained with ethidium bromide, and digitally photographed (AlphaImager, Alpha Innotech Co, San Leandro, CA, USA) in a UV light. A 100-bp ladder (Amersham Biosciences) served as a molecular weight marker (**IV, V**).

Statistical methods (II, III, V, unpublished)

From the PCR-RFLP banding patterns, a distance matrix was calculated with the NTSYS program (Numerical Taxonomy and Multivariate Analysis System; Applied Biostatistics Inc., Setauket, New York, USA) using Dice coefficient (also named Nei and Li coefficient), where the presence or absence of a band was used as a character (Weisburg *et al.* 1991). The distance matrix was plotted as a phenogram using UPGMA clustering (**II**). Student's T-test and Tukeys ANOVA were used for comparing the strength of hemagglutination (**III**). Chi-square (χ^2) test was used to evaluate the significance of clonal persistence and non-linear simple regression (curve-fitting) was used for evaluating the relationship between the number of isolates investigated and the clonal types found (**V**). From the AP-PCR banding patterns, a distance matrix was calculated with the BioNumerics program (Applied Maths BVBA, Sint-Martens-Latem, Belgium) using Dice coefficient, where the presence or absence of a band was used as a character (Weisburg *et al.* 1991). One way ANOVA was used to compare the clonal types between individual infants (**unpublished**).

RESULTS

Identification scheme (I)

Using the selected 5 simple phenotypic tests (Gram staining, direct UV fluorescence, MUG, CAAM, and hemagglutination) for identification of pigmented Gram-negative isolates, all reference strains and clinical isolates of *P. gingivalis* were correctly identified to the species level. The phenotypic screening also separated the *P. intermedia*/*nigrescens* group from the *P. melaninogenica* group but no further separation was possible with these tests. Of the 25 isolates which remained unidentified with the screening, 11 resembled *P. melaninogenica*, except that they demonstrated hemagglutinating ability. The API 20A and rapid ID 32A commercial kits (BioMérieux) identified 9 isolates to other species than *P. intermedia*, *P. melaninogenica* or *P. gingivalis*: 3 isolates as *P. oralis*, 2 isolates as *Veillonella* spp., 2 isolates as *Bacteroides ureolyticus*, one isolate as *Bacteroides capillosus*, and one isolate as the Gram-positive *Bifidobacterium adolescentis*. Furthermore, the kits were able to identify 13 isolates that the phenotypic screening could not: 8 isolates were hemagglutinating *P. melaninogenica*, 2 isolates *P. intermedia* (one slightly hemagglutinating, the other MUG positive), 2 isolates *P. oralis*, and one isolate *B. ureolyticus*. Sixteen of the 30 isolates (53%) identified as *P. intermedia* with the kits proved to be *P. nigrescens* with PCR. Due to β-galactosidase activity, 8 isolates were identified as *P. melaninogenica* with the screening but proved to be *P. intermedia* (3 isolates) or *P. nigrescens* (5 isolates) when tested with PCR.

The commercial kits identified only 58% (API 20A) and 28% (rapid ID 32A) of the clinical isolates to the species level. Neither of the kits could separate *P. nigrescens* from *P. intermedia*, and the API 20A kit failed to identify *P. gingivalis*. In addition, the type strains of *P. loescheii*, *P. asaccharolytica*, and *F. nucleatum* were not correctly identified with either of the kits. The 16S rDNA PCR method correctly identified all *P. intermedia*, *P. nigrescens*, *P. melaninogenica*, and *P. gingivalis* reference strains but the type strain of *P. veroralis* was incorrectly identified as *P. melaninogenica*. No cross-reaction was found among the other species.

Taxonomic status of isolates of the *P. melaninogenica* group (II)

The 16S rDNA PCR method using the fD1/rD1 primers gave bands, approximately 1512 nucleotides in size, from all tested isolates. Digested DNA gave 2-9 bands. At 70% similarity, 6 clusters were formed, and 7 strains did not fall into a cluster with any other

strain or isolate (all were type strains of separate *Prevotella* species). One cluster included 2 type strains (*P. veroralis* and *P. melaninogenica*). The hemagglutinating isolates resembling *P. melaninogenica* did not form a single homologous group but fell into 3 clusters: 4 isolates into a cluster with the type strains of *P. melaninogenica* and *P. veroralis*, 4 isolates into a cluster with clinical isolates only, and 2 isolates into a cluster with the type strain of *P. loescheii*.

Properties of hemagglutination by *P. melaninogenica* (II, III)

In electron microscopy, fimbria-like structures were clearly apparent on the cells of young cultures of the hemagglutinating *P. melaninogenica* group isolates. These structures were not seen on non-hemagglutinating cells. The strength of the hemagglutination of *P. melaninogenica* was significantly less than that of *P. gingivalis*. The *P. melaninogenica* hemagglutination was inhibited by raffinose, galactose, and lactose, but no sugar tested had any effect on *P. gingivalis* hemagglutination. The *P. melaninogenica* hemagglutination was eliminated by heating at 80°C for 30 min and was reduced by proteinase digestion of the bacteria and shaking of the bacteria. The supernatant of shaken bacterial cells had, however, the same hemagglutinating ability as non-shaken cells.

AP-PCR typing (IV, V)

Out of the 12 primers tested for AP-PCR typing, 8 primers resulted in poor amplification using the type strains of *F. nucleatum* subspp., whereas 4 primers, C1, C2, D8635, and D11344, revealed unique and reproducible fingerprints. The amplification patterns of the clinical isolates generally consisted of 2-5 major amplicons. The isolates sharing an amplification pattern derived from one primer usually shared the patterns constructed with the other 3 primers. All isolates were typed using at least 2 primers. Occasionally, isolates were found to be identical with one primer but dissimilar with the other. These were subjected to amplification with the other 2 primers and in all cases were also separated by the additional primers.

Genetic diversity within oral *F. nucleatum* populations (IV, unpublished)

The relationship between the total number of *F. nucleatum* isolates from each sample examined and the number of AP-PCR types found among these isolates indicated that at least 10-20 isolates are needed to view the actual clonal diversity within a sample.

Each infant harbored 5-14 (mean 8.3) different AP-PCR types during their 2 first years of life. Up to 7 AP-PCR types could be found in one sample. At each time, usually one AP-PCR type was dominating the other types: a single AP-PCR type accounted for ≥50% of the available isolates in 36 of the 45 samples (80%), and in the remaining 9 samples the dominant type represented at least a third of the available isolates.

Usually, infants harbored their own separate *F. nucleatum* AP-PCR types, only one AP-PCR type was found in 2 separate infants. An AP-PCR type was not more related to other AP-PCR types in the same infant than to AP-PCR types of the other infants.

Clonal persistence of oral *F. nucleatum* during the first 2 years (IV)

At least one AP-PCR type was found to persist for up to one year in 11 of the 12 infants examined. In one infant, all AP-PCR types were replaced in the subsequent samples, each collected 6 months apart. During the first year of life, only 22% of AP-PCR types persisted but the persistence of strains became more common during the second year of life when 44% of AP-PCR types persisted, although the difference was not statistically significant. The dominant AP-PCR types were not more likely to be found in saliva collected on the next sampling than the other types, nor were the persistent AP-PCR types more likely to be dominating in the subsequent salivary sample.

Origin of nasopharyngeal *F. nucleatum* (V)

Identical AP-PCR types were found among salivary and nasopharyngeal *F. nucleatum* isolates in 5 of the 8 infants examined. In one infant, AP-PCR typing revealed an identical pattern between *F. nucleatum* collected from saliva at the healthy visit at 12 months of age and the isolate from the nasopharynx during an AOM episode 2 months later. In 3 infants, the nasopharyngeal *F. nucleatum* isolate shared an identical AP-PCR pattern with salivary *F. nucleatum* isolates collected 1-3.5 months after their AOM episodes. In one infant, an identical AP-PCR pattern was found among *F. nucleatum* isolates collected from the nasopharynx and saliva at the same scheduled visit at 18 months of age.

DISCUSSION

Methodological considerations

Samples and isolates (**I, II, IV, V**)

Traditionally, subgingival samples have been taken with either a curette or paperpoint and some variations have been documented between and within these sampling methods (Hartroth *et al.* 1999, Tanner and Goodson 1986). Sampling by paperpoint is less invasive than by curette but may result in an underestimation of tightly adherent bacteria in subgingival sites (Hartroth *et al.* 1999). Lately, saliva has been used as a suitable specimen for oral Gram-negative anaerobes as it can be collected in an easy non-invasive way. Comparable frequencies of oral anaerobes and similar genotypes have been found in saliva and subgingival plaque samples (Mättö *et al.* 1996a). Therefore, saliva gives a good insight into the overall microbial world of the oral cavity. Furthermore, saliva offers the most plausible vehicle for intra-individual translocation of oral bacteria to close anatomical sites, such as the nasopharynx. In the present study (**IV, V**), unstimulated saliva was collected from the buccal area of the mouth, whereas NP and NPA samples were collected through the nasal cavity, thus excluding salivary contamination.

For culture, an ideal transport medium keeps the microbes alive and preserves their proportions in the sample. Anaerobiosis and low redox potential of the transport media is essential for survival of anaerobes (Dahlén *et al.* 1993). The most commonly used transport media are reduced transport fluid (Syed and Loesche 1972) and variations of the VMG transport media (Möller 1966). In the present study, the VMG II (**I, II**) or VMGA III (**IV, V**) was used to transport samples from the study clinic to the laboratory. Both VMG media have been demonstrated to support the survival of anaerobic bacteria for 1-2 days (Dahlén *et al.* 1993).

The number of isolates to be picked up from a microbiological sample depends on the aim of the study. In the present study (**IV**), the aim was to get an overview of the actual clonal diversity within developing *F. nucleatum* populations in the oral cavity. Because *S. mitis* biovar 1 (Hohwy and Kilian 1995) and *P. melaninogenica* (Könönen *et al.* 1994a, Könönen *et al.* 1994c), which are among the early colonizers of the mouth, have a wide clonal heterogeneity intra-individually, we estimated that a large number of isolates might be required to find the actual number of *F. nucleatum* clones present in the sample. The present study on salivary *F. nucleatum* (**IV**) demonstrated that if less than 10 isolates of *F. nucleatum* were examined, the number of genotypes were most likely

underestimated, whereas the examination of 20-25 isolates per sample was likely to reveal the actual clonal diversity within this bacterial population. This confirms the similar conclusions on *S. mitis* biovar 1 by Hohwy *et al.* (2001) and is well in line with the view on the wide clonal diversity within early-colonizing oral commensals.

Adhesion assays (**II, III**)

Similar to *Escherichia coli* (Goldhar 1994), different specificity in agglutination of erythrocytes from different animal species has seen with some *Prevotella* species (Leung *et al.* 1989, Okamoto *et al.* 1999, Weiss *et al.* 1989). After comparing the ability of *P. melaninogenica* and *P. gingivalis* strains to agglutinate erythrocytes from 6 animal species, human blood was selected for routine testing because of its easy availability (**III**). The substantial natural variability of glycoproteins and glycolipids on the surface of erythrocytes makes them a convenient model for evaluating the specificity of bacterial adherence. Hemagglutination tests can be performed using microscopic slides, which offer fast results, test tubes, or microtiter plates, which are considered most convenient for inhibition assays (Goldhar 1994, Goldhar 1995).

DNA isolation (**I, II, IV, V**)

For gene amplification, chelating resins have been widely used in DNA extraction procedures from bacterial and viral (de Lamballerie *et al.* 1992), fungal (Möhlenhoff *et al.* 2001), and human (Walsh *et al.* 1991) origin. Chelex 100 (Bio-Rad) can chelate a large amount of the divalent metal ions, for which PCR is especially sensitive and it can be easily removed, allowing the Mg^{++}-dependent PCR DNA amplification (Walsh *et al.* 1991). This resin has been reported to increase the sensitivity of DNA amplification, probably by minimizing inhibitory molecules present in original samples (Jaulhac *et al.* 1998, Mathis *et al.* 1997, Mättö *et al.* 1998). According to our experience (**I**), Chelex extraction is simple, inexpensive, and time-saving compared to the time-consuming and cumbersome phenol-chloroform extraction.

Classification using 16S rDNA PCR-RFLP (**II**)

The rationale of RFLP analysis is that the genetic difference between DNA sequences correlates with the proportion of DNA fragments shared by them, i.e. the closer the cleavage patterns, the higher the similarity (Jensen *et al.* 2002). RFLP analysis of PCR-

amplified 16S rDNA has been found to be an appropriate technique for differentiation and characterization of microorganisms (Ruiz *et al.* 2000). The minimum number of restriction enzymes needed to examine the phylogenetic relationship of various isolates is an important factor. In 16S rDNA PCR-RFLP, most reliable estimations seem to originate from combinations of 3 or more tetrameric (i.e. having 4-bp recognition sites) restriction enzyme, as demonstrated by computer simulation (Moyer *et al.* 1996) and confirmed by RFLP experiments (Laguerre *et al.* 1996, Urakawa *et al.* 1997). In the present study (**II**), 5 tetrameric restriction enzymes were used, resulting in high differentiation among the 43 strains tested, where only 2 *P. oralis* strains shared identical RFLP pattern. As other authors have reported (Jang *et al.* 2003), the PCR-RFLP technique was found to be technically less demanding than most other molecular-biological approaches and required only a simple DNA extraction procedure.

Clonal typing using AP-PCR (**IV, V**)

AP-PCR is a commonly used method for clonal typing of various oral microorganisms, including Gram-negative anaerobic rods (Fukui *et al.* 1999, Mättö *et al.* 1996a, Ménard and Mouton 1995, van Steenbergen *et al.* 1993a), Gram-negative facultatives (Chen and Ashimoto 1996, Dogan *et al.* 1999, Fujise *et al.* 2004, Kaplan *et al.* 2002, Paju *et al.* 2000, Saarela *et al.* 1999), viridans streptococci (Grönroos and Alaluusua 2000, Li and Caufield 1998, Li *et al.* 2001, Pan *et al.* 2001, Redmo Emanuelsson *et al.* 2003), Gram-positive facultative rods (Ruby *et al.* 2002), and *Candida* species (Hannula *et al.* 1999). However, only few researchers have used this method for the differentiation of *F. nucleatum* (Avila-Campos *et al.* 1999, George *et al.* 1997, Moraes *et al.* 2002). Different primers have different discriminatory power (George *et al.* 1997, Mättö *et al.* 1996a, Ménard and Mouton 1995), thus emphasizing the importance of using more than one primer for AP-PCR analysis of bacteria. In the present study (**VI, V**), after testing 12 primers using 4 reference strains of *F. nucleatum*, 4 primers were selected for AP-PCR typing of clinical *F. nucleatum* isolates in order to eliminate the need for further confirmation. These 4 primers have previously been reported to produce discriminating AP-PCR patterns for different *Fusobacterium* strains (George *et al.* 1997, Narongwanichgarn *et al.* 2001).

Several methodological factors affect the outcome of AP-PCR, such as the type of thermal cycler and concentration of the template, primer, and polymerase (Meunier and Grimont 1993, Tyler *et al.* 1997). Some run-to-run variations have been reported in AP-

PCR (Fukui *et al.* 1999), whereas other authors have found a good inter-assay reproducibility (van Steenbergen *et al.* 1993a). This stresses the importance of standardization and validation of all parameters. In the present study (**VI, V**), the commercial Ready-To-Go-PCR kit (Amersham Biosciences) was used, always in the same thermal cycler, in order to achieve quality standardization. Using these standardization methods, AP-PCR typing proved to be a simple, rapid, and reproducible method for differentiating *F. nucleatum* isolates, confirming the experience of George *et al.* (1997).

Identification of Gram-negative anaerobes (I, unpublished)

The screening with rapid tests is a sufficient method for identifying *P. gingivalis* to the species level and categorize *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* to their appropriate groups (**I**). Addition of the indole spot test (Jousimies-Somer *et al.* 2002) would increase the accuracy of our simple identification scheme by recognizing occasional β-galactosidase (MUG) positive *P. intermedia*/*nigrescens* isolates otherwise misidentified as to belong to the *P. melaninogenica* group. Other authors have also reported lactose-fermenting strains among *P. intermedia* and *P. nigrescens* (Dahlén *et al.* 1990, Fukushima *et al.* 1992) as well as among *P. pallens* (Könönen *et al.* 1998b).

Commercial identification kits did not improve the identification achievable with the phenotypic screening for the 4 species examined. *P. gingivalis*, which was easily identified with simple phenotypic tests, was usually not identifiable with the kits because they were completely unreactive in the API kit. Furthermore, *P. melaninogenica*-like strains remained frequently unidentified to the species level and neither of the kits was able to differentiate *P. nigrescens* from *P. intermedia* (**I**). Commercial kits are expensive, somewhat time-consuming and seem to offer no assistance in identification at this level.

The 16S rDNA PCR proved to be an accurate, relatively straightforward, and reproducible method for identification of *P. gingivalis* and separation of *P. nigrescens* from *P. intermedia* (**I**). Similar methods have been used by other laboratories with a good success (Ashimoto *et al.* 1996, Conrads *et al.* 1997, Conrads *et al.* 1999, García *et al.* 1998, Gmür and Thurnheer 2002, Kuboniwa *et al.* 2004, Martin *et al.* 2002, Mättö *et al.* 1996a, Okamoto *et al.* 1999, Premaraj *et al.* 1999, Riggio *et al.* 1998, Shah *et al.* 1995b, Slots *et al.* 1995, Stubbs *et al.* 1999). Other genes have been targeted with species-specific PCR such as the *P. gingivalis* fimbrial (*fimA*) (Doungudomdacha *et al.* 2000) and Arg-

gingipain (Morillo *et al.* 2003) genes, and *P. intermedia* acid phosphatase (*phoC*), *P. melaninogenica* hemolysin (*phyA*), and *P. loescheii* adhesin precursor (*plaA*) genes (Yoshida *et al.* 2005). In contrast, identification of *P. melaninogenica* with 16S rDNA based PCR was problematical because of high 16S rDNA sequence similarity with *P. veroralis*.

Earlier investigations associated the former *Bacteroides intermedius*, currently including *P. intermedia* and *P. nigrescens*, with hormone-induced pregnancy gingivitis (Kornman and Loesche 1980). With the aim to determine whether *P. intermedia* or *P. nigrescens* or both species are involved in the subgingival microbial shift during pregnancy, the 16S rDNA PCR method was used to identify the *P. intermedia*/*nigrescens* group isolates collected from subgingival plaque and saliva of 28 pregnant women. All the women had symptoms of pregnancy gingivitis, but were in other ways periodontally healthy (Latva-aho *et al.* 2004). Of the 1109 isolates tested, 982 (89%) were identified as *P. nigrescens* but only 8 isolates from 2 women as *P. intermedia* **(unpublished)** indicating that *P. nigrescens* is associated with pregnancy gingivitis. This is in line with the observation (Mättö *et al.* 1996b) on the common presence of *P. nigrescens* and the absence of *P. intermedia* in relatively young Finnish subjects without advanced periodontitis. The remaining 119 isolates could not be identified as either of the 2 species. These isolates could possibly be of a related species for which we had no primers available, for example *P. tannerae*, as other authors have experienced (Xia *et al.* 2000).

Significance of finding hemagglutinating *P. melaninogenica* (II, III)

Hemagglutination often seems to be linked with fimbria (Chandad and Mouton 1995, Leung *et al.* 1999) and, in combination with hemolysis, may contain potential pathogenic mechanisms involved in oral infections. *P. gingivalis* and *P. intermedia*, which have been linked to periodontal diseases (Consensus report 1996), are fimbriated and able to agglutinate erythrocytes (Leung *et al.* 1996, Ogawa and Hamada 1994, Okamoto *et al.* 1999). The present study (**II, III**) showed the presence of hemagglutinating bacteria closely resembling *P. melaninogenica*, in patients with periodontitis. Some association between the isolation of hemagglutinating strains of *P. melaninogenica* and periodontitis was initially seen, but when a larger collection of *P. melaninogenica* was investigated, this association was lost (**unpublished**). The hemagglutinating strength of *P. melaninogenica* proved to be far less than that found in the major periodontal pathogen *P. gingivalis*. The

hemagglutinating agent of *P. melaninogenica* seemed to be a protein, which could be separated from the bacterial cell. It binds to raffinose, lactose, and galactose-containing carbohydrate residues on erythrocytes, unlike the hemagglutinating agent of *P. intermedia*, which binds to glucosamine-containing carbohydrates (**III**). Lactose and galactose-mediated hemagglutination of *P. melaninogenica* suggests a structural relation to the hemagglutinating mechanism of the related species *P. loescheii* (Weiss *et al.* 1989) and possibly also that of *F. nucleatum* (Gaetti-Jardim and Avila-Campos 1999). Since L-arginin and the proteinase inhibitor antipain inhibited the agglutination of *P. gingivalis* but had no effect on hemagglutination of *Prevotella*, this indicates that the hemagglutinating activity of *Prevotella* is not mediated in connection with proteinases, unlike that of *P. gingivalis* (Nishikata and Yoshimura 1991, Shah *et al.* 1992, Yoneda and Kuramitsu 1996). As the potential virulence factors of the hemagglutinating variants of *P. melaninogenica* are significantly weaker than those of more potential periodontal pathogens, these variants may only be favored by conditions created in disease, rather than having a role as an opportunistic pathogen.

The hemagglutinating isolates resembling *P. melaninogenica* did not form a single homologous group, but fell into 3 clusters: with the *P. melaninogenica* and *P. veroralis* reference strains, with other clinical isolates, and with the *P. loescheii* reference strain (**II**). The division of the clinical *P. melaninogenica* isolates into 2 main clusters could be due to the 2 DNA homology groups of this species (Tanner *et al.* 1994) or some of the recently described, phenotypically similar species (Downes *et al.* 2005, Sakamoto *et al.* 2005).

Oral *F. nucleatum* populations (IV)

Each genetic variant of the oral commensal microbiota may express specific characters related to survival and extended persistence. *F. nucleatum* is a heterogeneous species and numerous AP-PCR profiles and high heterogeneity of serovars and ribotypes have been found within individuals (George *et al.* 1997, Thurnheer *et al.* 1999). The present study (**IV**) demonstrated a wide genetic diversity within oral *F. nucleatum* populations both intra-individually (up to 7 AP-PCR types/subject at a time) and between individuals (only one AP-PCR type found in more than one infant). In general, the early-colonizing commensals with a wide antigenic variety can elicit natural immunity that is considered to be a benefit to the host (Smith *et al.* 1998). The clonal heterogeneity and frequent turnover

of clones among oral *F. nucleatum* populations intra-individually allows the species to escape the host immune response, and thus persistently colonize the oral cavity. The persistence of the species might, furthermore, be beneficial for the host, as it stimulates the natural immune response. Although the emergence and disappearance of different genotypes could be due to mutations or genetic recombination, Hohwy *et al.* (2001) rejected that hypothesis in their recent study on *S. mitis* biovar 1. The present study (**unpublished**) supports that conclusion, since the *F. nucleatum* AP-PCR types of each infant were not more related to the other AP-PCR types in the same infant than to AP-PCR types of the other infants.

In 11 of the 12 infants examined, identical AP-PCR types were found on subsequent sampling occasions, and they were persistent at least for up to one year. Similarly, mutans streptococci have been found to persist for up to many years, both in children and adults (Alaluusua *et al.* 1994, Köhler *et al.* 2003, Redmo Emanuelsson and Thornqvist 2000), and clonal persistence has been demonstrated for months for *P. intermedia* and *P. nigrescens* (Teanpaisan *et al.* 1996) and for years for *A. actinomycetemcomitans* (Saarela *et al.* 1999) in adult subjects. In contrast, no persistent genotypes of *S. mitis* biovar 1 could be detected in the 2 examined infants who at the end of the 9-10 month follow-up period were 19- and 15-months-old (Hohwy *et al.* 2001) and, in adults, no clone of *F. nucleatum* was found to persist over a 16-month period (Suchett-Kaye *et al.* 1998), and only few *E. corrodens* clones persisted for 9 months (Fujise *et al.* 2004). In the present study (**IV**), although some AP-PCR types were persistent, the majority of the *F. nucleatum* population was constantly changing; distinct AP-PCR types emerged and disappeared, and a high variability was seen in the proportions of persistent types throughout the following period. Persistence of *F. nucleatum* AP-PCR types in saliva was occasional during the first year of life; however, persistent types became more frequent after one year of age. Due to eruption of primary dentition, a new microbial habitat, the gingival crevice, offers an optimal habitat for many anaerobic species colonizing the oral cavity. This improved living environment may explain the increased persistence of *F. nucleatum* clones with age. However, Suchett-Kaye *et al.* (1998) found no persisting ribotypes among 38 *F. nucleatum* isolates from 8 adult dental students compared to 61 isolates collected 16 months earlier.

Origin of nasopharyngeal *F. nucleatum* (V)

In children, *F. nucleatum* has been associated with infections in the head and neck area (Brook 1994). The species presents some properties, which are regarded as virulence factors, such as binding to epithelial cells and invading them (Han *et al.* 2000). However, these properties may vary between different clones. For example, β-lactamase-producing and non-producing *F. nucleatum* strains can be simultaneously isolated from young children (Könönen *et al.* 1999b, Nyfors *et al.* 2003). Furthermore, *F. nucleatum* has been found as the most common anaerobic finding (Könönen *et al.* 2003) in nasopharyngeal aspirates collected from infants during acute otitis media (part of these included in the present study). Interestingly, the colonization of infants' nasopharynges by anaerobes occurs transiently during infection (Könönen *et al.* 2003). These observations led to the research on the potential source of nasopharyngeal anaerobes. In the present study (**V**) identical AP-PCR types were found between the nasopharyngeal and salivary isolates from the same infant in 5 of the 8 examined infants, thus indicating that the source of transient colonization of nasopharyngeal anaerobes is the oral cavity and saliva their transmission vehicle. Whether *F. nucleatum* colonizes the nasopharynx just because of ecological changes favoring its growth or whether it could play an active role in the biofilm formation on nasopharyngeal mucosa and in pathogenesis of AOM is not known.

F. nucleatum is frequently found in middle ear effusion from children with otitis media with effusion (Brook *et al.* 2000). Part of the *F. nucleatum* isolates from the oral cavity of infants belonging to the FinOM cohort study produced β-lactamase (Nyfors *et al.* 2003). In this study (**unpublished**) no AP-PCR types were found including both β-lactamase-producing and non-β-lactamase-producing isolates. Furthermore, some β-lactamase-producing strains isolated from the oral cavity, were found in the nasopharynx of the same infant (**V**). In case anaerobic bacteria, such as *F. nucleatum*, are involved in the pathogenesis of AOM, their existence in the nasopharynx may have an impact on the treatment of these common pediatric infections.

SUMMARY AND CONCLUSIONS

This study was focused on common anaerobic Gram-negative bacteria that are generally considered to belong to the commensal microbiota, even though some strains may possess pathogenic potential. The present study 1) evaluated different methods for identification of selected oral anaerobes; 2) viewed the population structure and dynamics of an early-colonizing oral commensal; 3) clarified the pathogenic potential of common oral commensals and; 4) demonstrated translocation of an oral species to the nasopharynx, in connection with respiratory infections.

Acceptance/rejection of hypotheses:

- The working hypothesis that hemagglutinating *P. melaninogenica* is a separate species with hemagglutination similar to that of *P. gingivalis* was rejected.
- The working hypothesis on the high clonal diversity and high turnover rate of clones among oral *F. nucleatum* populations was accepted. However, during the second year of life clonal stability increases.
- The working hypothesis on the oral origin of anaerobic bacteria colonizing the nasopharynx related to respiratory infection was accepted.

Key findings and main conclusions:

I. Phenotypic screening was valuable for identifying *P. gingivalis* and differentiated lactose-fermenting species from non-fermenting species. Commercial identification kits tested failed to improve the level of identification achieved with the phenotypic screening. Neither the kits nor the phenotypic screening could differentiate *P. intermedia* and *P. nigrescens*, whereas the 16S rDNA PCR method easily separated the species. The PCR method increases the reliability of identification of a range of Gram-negative anaerobic bacteria.

II. A hemagglutinating variant of *P. melaninogenica* appeared fimbriated when viewed in electron microscope. PCR-RFLP results showed that the hemagglutinating strains did not form a homologous group inside the *Prevotella* genus but fell into 3 distinct clusters. Two main clusters may be due to the 2 DNA homology groups found within the species or they may correlate to the recently described species of *Prevotella*.

III. The strength of *P. melaninogenica* hemagglutination was considerably less than that of *P. gingivalis*. The hemagglutinating agent on *P. melaninogenica* seemed to be a protein, which could be separated from the cell and could bind to lactose, galactose, and raffinose-containing carbohydrates on the erythrocytes. This potential virulence factor of *P. melaninogenica* is of a significantly lower magnitude than that of major periodontal pathogens, and this hemagglutinating variant of *P. melaninogenica* is, at most, scarcely pathogenic.

IV. A wide genetic diversity was seen within oral *F. nucleatum* populations in infants from whom up to 7 AP-PCR types could be simultaneously detected at a time. This high clonal heterogeneity combined with frequent turnover of clones might allow the species to escape the host immune response, and persistently to colonize the oral cavity. Strain turnover rate was high during the first year of life, but then persistent clones were increasingly found. In 11 of the 12 infants examined, AP-PCR types persisted for up to one year.

V. In 5 of the 8 infants examined, identical AP-PCR types were found between the nasopharyngeal and salivary isolates. Since anaerobes seem to be only transiently present in the nasopharynx and salivary contamination of the nasopharyngeal samples could be excluded, this observation indicates that the source of nasopharyngeal anaerobes was the oral cavity and saliva the transmission vehicle.

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REFERENCES

Agnani G, Tricot-Doleux S, Du L, Bonnaure-Mallet M. Adherence of *Porphyromonas gingivalis* to gingival epithelial cells: modulation of bacterial protein expression. Oral Microbiol Immunol 2000; 15: 48-52.

Alaluusua S, Saarela M, Jousimies-Somer H, Asikainen S. Ribotyping shows intrafamilial similarity in *Actinobacillus actinomycetemcomitans* isolates. Oral Microbiol Immunol 1993; 8: 225-229.

Alaluusua S, Alaluusua SJ, Karjalainen J, Saarela M, Holttinen T, Kallio M, Holtta P, Torkko H, Relander P, Asikainen S. The demonstration by ribotyping of the stability of oral *Streptococcus mutans* infection over 5 to 7 years in children. Arch Oral Biol 1994; 39: 467-471.

Ali RW, Skaug N, Nilsen R, Bakken V. Microbial associations of 4 putative periodontal pathogens in Sudanese adult periodontitis patients determined by DNA probe analysis. J Periodontol 1994; 65: 1053-1057.

Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun 1997; 65: 2765-2771.

Andersson DI. Persistence of antibiotic resistant bacteria. Curr Opin Microbiol 2003; 6: 452-456.

Arzese AR, Tomasetig L, Botta GA. Detection of tetQ and ermF antibiotic resistance genes in *Prevotella* and *Porphyromonas* isolates from clinical specimens and resident microbiota of humans. J Antimicrob Chemother 2000; 45: 577-582.

Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996; 11: 266-273.

Asikainen S, Chen C, Slots J. Likelihood of transmitting *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in families with periodontitis. Oral Microbiol Immunol 1996; 11: 387-394.

Avgustin G, Wallace RJ, Flint HJ. Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. Int J Syst Bacteriol 1997; 47: 284-288.

Avila-Campos MJ, Sacchi CT, Whitney AM, Steigerwalt AG, Mayer LW. Arbitrarily primed-polymerase chain reaction for identification and epidemiologic subtyping of oral isolates of *Fusobacterium nucleatum*. J Periodontol 1999; 70: 1202-1208.

Bachrach G, Rosen G, Bellalou M, Naor R, Sela MN. Identification of a *Fusobacterium nucleatum* 65 kDa serine protease. Oral Microbiol Immunol 2004; 19: 155-159.

Bailit HL, Baldwin DC, Hunt EE, Jr. The increasing prevalence of gingival *Bacteroides melaninogenicus* with age in children. Arch Oral Biol 1964; 9: 435-438.

Beck JD, Offenbacher S. The association between periodontal diseases and cardiovascular diseases: a state-of-the-science review. Ann Periodontol 2001; 6: 9-15.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002; 40: 1001-1009.

Beem JE, Nesbitt WE, Leung KP. Identification of hemolytic activity in *Prevotella intermedia*. Oral Microbiol Immunol 1998; 13: 97-105.

Beem JE, Nesbitt WE, Leung KP. Cloning of *Prevotella intermedia* loci demonstrating multiple hemolytic domains. Oral Microbiol Immunol 1999; 14: 143-152.

Bolstad AI, Jensen HB, Bakken V. Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. Clin Microbiol Rev 1996; 9: 55-71.

Bradshaw DJ, Marsh PD, Allison C, Schilling KM. Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. Microbiology 1996; 142 (Pt 3): 623-629.

Bradshaw DJ, Marsh PD, Watson GK, Allison C. Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. Infect Immun 1998; 66: 4729-4732.

Brook I, Walker RI. The relationship between Fusobacterium species and other flora in mixed infection. J Med Microbiol 1986; 21: 93-100.

Brook I. The role of anaerobic bacteria in otitis media: microbiology, pathogenesis, and implications on therapy. Am J Otolaryngol 1987; 8: 109-117.

Brook I, Frazier EH. Aerobic and anaerobic microbiology of empyema. A retrospective review in two military hospitals. Chest 1993; 103: 1502-1507.

Brook I. Fusobacterial infections in children. J Infect 1994; 28: 155-165.

Brook I. *Prevotella* and *Porphyromonas* infections in children. J Med Microbiol 1995; 42: 340-347.

Brook I. Isolation of non-sporing anaerobic rods from infections in children. J Med Microbiol 1996; 45: 21-26.

Brook I, Yocum P, Shah K. Aerobic and anaerobic bacteriology of concurrent chronic otitis media with effusion and chronic sinusitis in children. Arch Otolaryngol Head Neck Surg 2000; 126: 174-176.

Carlsson J, Grahnen H, Jonsson G, Wikner S. Establishment of *Streptococcus sanguis* in the mouths of infants. Arch Oral Biol 1970a; 15: 1143-1148.

Carlsson J, Grahnen H, Jonsson G, Wikner S. Early establishment of *Streptococcus salivarius* in the mouth of infants. J Dent Res 1970b; 49: 415-418.

Casadevall A, Pirofski L-a. Host-Pathogen Interactions: Redefining the Basic Concepts of Virulence and Pathogenicity. Infect Immun 1999; 67: 3703-3713.

Casadevall A, Pirofski L-a. Host-Pathogen Interactions: Basic Concepts of Microbial Commensalism, Colonization, Infection, and Disease. Infect Immun 2000; 68: 6511-6518.

Caufield PW, Walker TM. Genetic diversity within Streptococcus mutans evident from chromosomal DNA restriction fragment polymorphisms. J Clin Microbiol 1989; 27: 274-278.

Chandad F, Mouton C. Antigenic, structural, and functional relationships between fimbriae and the hemagglutinating adhesin HA-Ag2 of *Porphyromonas gingivalis*. Infect Immun 1995; 63: 4755-4763.

Chen C, Ashimoto A. Clonal diversity of oral *Eikenella corrodens* within individual subjects by arbitrarily primed PCR. J Clin Microbiol 1996; 34: 1837-1839.

Choi BK, Paster BJ, Dewhirst FE, Göbel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun 1994; 62: 1889-1895.

Choi J, Borrello MA, Smith E, Cutler CW, Sojar H, Zauderer M. Prior exposure of mice to *Fusobacterium nucleatum* modulates host response to *Porphyromonas gingivalis*. Oral Microbiol Immunol 2001; 16: 338-344.

Chryssagi AM, Brusselmans CB, Rombouts JJ. Septic arthritis of the hip due to *Fusobacterium nucleatum*. Clin Rheumatol 2001; 20: 229-231.

Chu L, Bramanti TE, Ebersole JL, Holt SC. Hemolytic activity in the periodontopathogen *Porphyromonas gingivalis*: kinetics of enzyme release and localization. Infect Immun 1991; 59: 1932-1940.

Collins MD, Love DN, Karjalainen J, Kanervo A, Forsblom B, Willems A, Stubbs S, Sarkiala E, Bailey GD, Wigney DI, Jousimies-Somer H. Phylogenetic analysis of members of the genus *Porphyromonas* and description of *Porphyromonas cangingivalis* sp. nov. and *Porphyromonas cansulci* sp. nov. Int J Syst Bacteriol 1994; 44: 674-679.

Conrads G, Pelz K, Hughes B, Seyfarth I, Devine DA. Optimized oligonucleotides for the differentiation of *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1997; 12: 117-120.

Conrads G, Flemmig TF, Seyfarth I, Lampert F, Lutticken R. Simultaneous detection of *Bacteroides forsythus* and *Prevotella intermedia* by 16S rRNA gene-directed multiplex PCR. J Clin Microbiol 1999; 37: 1621-1624.

Conrads G, Claros MC, Citron DM, Tyrrell KL, Merriam V, Goldstein E. 16S-23S rDNA internal transcribed spacer sequences for analysis of the phylogenetic relationships among species of the genus *Fusobacterium*. Int J Syst Evol Microbiol 2002; 52: 493-499.

Conrads G, Citron DM, Tyrrell KL, Horz HP, Goldstein EJ. 16S-23S rRNA gene internal transcribed spacer sequences for analysis of the phylogenetic relationships among species of the genus *Porphyromonas*. Int J Syst Evol Microbiol 2005; 55: 607-613.

Consensus report. Periodontal diseases: pathogenesis and microbial factors. Ann Periodontol 1996; 1: 926-932.

Costerton JW, Cook G, Lamont R. The community architecture of biofilms: dynamic structures and mechanisms. In: Dental plaque revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 5-14.

Dahlén G, Wikström M, Renvert S, Gmür R, Guggenheim B. Biochemical and serological characterization of *Bacteroides intermedius* strains isolated from the deep periodontal pocket. J Clin Microbiol 1990; 28: 2269-2274.

Dahlén G, Möller ÅJ. Microbiology of endodontic infections. In: Contemporary Oral Microbiology and Immunology. Slots J, Taubman MA (eds). 1992; St. Louis: Mosby Year Book, 444-475.

Dahlén G, Pipattanagovit P, Rosling B, Möller ÅJ. A comparison of two transport media for saliva and subgingival samples. Oral Microbiol Immunol 1993; 8: 375-382.

Dahlén GG, Johnson JR, Gmür R. *Prevotella intermedia* and *Prevotella nigrescens* serotypes, ribotypes and binding characteristics. FEMS Microbiol Lett 1996; 138: 89-95.

Darby I, Curtis M. Microbiology of periodontal disease in children and young adults. Periodontol 2000 2001; 26: 33-53.

Davenport ES, Williams CECS, Sterne JAC, Murad S, Sivapathasundram V, Curtis MA. Maternal Periodontal Disease and Preterm Low Birthweight: Case-Control Study. J Dent Res 2002; 81: 313-318.

de Lamballerie X, Zandotti C, Vignoli C, Bollet C, de Micco P. A one-step microbial DNA extraction method using "Chelex 100" suitable for gene amplification. Res Microbiol 1992; 143: 785-790.

de Soet JJ, Bokhout B, Buijs JF, van Loveren C, de Graaff J, Prahl-Andersen B. Transmission of mutans streptococci between mothers and children with cleft lip and/or palate. Cleft Palate Craniofac J 1998; 35: 460-464.

Debelian GJ, Olsen I, Tronstad L. Systemic diseases caused by oral microorganisms. Endod Dent Traumatol 1994; 10: 57-65.

Deshpande RG, Khan MB. Purification and characterization of hemolysin from *Porphyromonas gingivalis* A7436. FEMS Microbiol Lett 1999; 176: 387-394.

Devine DA, Pearce MA, Gharbia SE, Shah HN, Dixon RA, Gmür R. Species-specificity of monoclonal antibodies recognising *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1994; 120: 99-104.

Diaz PI, Zilm PS, Rogers AH. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. Microbiology 2002; 148: 467-472.

Dogan B, Asikainen S, Jousimies-Somer H. Evaluation of two commercial kits and arbitrarily primed PCR for identification and differentiation of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus*. J Clin Microbiol 1999; 37: 742-747.

Doungudomdacha S, Rawlinson A, Douglas CW. Enumeration of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples by a quantitative-competitive PCR method. J Med Microbiol 2000; 49: 861-874.

Downes J, Sutcliffe I, Tanner AC, Wade WG. *Prevotella marshii* sp. nov. and *Prevotella baroniae* sp. nov. isolated from the human oral cavity. Int J Syst Evol Microbiol 2005: in press.

Drucker DB, Gomes BP, Lilley JD. Role of anaerobic species in endodontic infection. Clin Infect Dis 1997; 25 (Suppl 2): S220-221.

Du L, Pellen-Mussi P, Chandad F, Mouton C, Bonnaure-Mallet M. Conservation of fimbriae and the hemagglutinating adhesin HA-Ag2 among *Porphyromonas gingivalis* strains and other anaerobic bacteria studied by epitope mapping analysis. Clin Diagn Lab Immunol 1997; 4: 711-714.

Dubreuil L, Behra-Miellet J, Vouillot C, Bland S, Sedallian A, Mory F. ß-lactamase production in *Prevotella* and in vitro susceptibilities to selected ß-lactam antibiotics. Int J Antimicrob Agents 2003; 21: 267-273.

Dymock D, Weightman AJ, Scully C, Wade WG. Molecular analysis of microflora associated with dentoalveolar abscesses. J Clin Microbiol 1996; 34: 537-542.

Dzink JL, Sheenan MT, Socransky SS. Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov.; *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov.; and *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. Int J Syst Bacteriol 1990; 40: 74-78.

Evaldson G, Heimdahl A, Kager L, Nord CE. The normal human anaerobic microflora. Scand J Infect Dis Suppl 1982; 35: 9-15.

Fiehn NE, Gutschik E, Larsen T, Bangsborg JM. Identity of streptococcal blood isolates and oral isolates from two patients with infective endocarditis. J Clin Microbiol 1995; 33: 1399-1401.

Finegold S, Jousimies-Somer H. Recently described clinically important anaerobic bacteria: medical aspects. Clin Infect Dis 1997; 25 (Suppl 2): S88-93.

Finegold SM, Vaisanen M-L, Rautio M, Eerola E, Summanen P, Molitoris D, Song Y, Liu C, Jousimies-Somer H. *Porphyromonas uenonis* sp. nov., a pathogen for humans distinct from *P. asaccharolytica* and *P. endodontalis*. J Clin Microbiol 2004; 42: 5298-5301.

Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of *Actinobacillus actinomycetemcomitans*. Periodontol 2000 1999; 20: 136-167.

Fournier D, Mouton C, Lapierre P, Kato T, Okuda K, Menard C. *Porphyromonas gulae* sp. nov., an anaerobic, gram-negative coccobacillus from the gingival sulcus of various animal hosts. Int J Syst Evol Microbiol 2001; 51: 1179-1189.

Frandsen EV, Poulsen K, Kilian M. Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. Int J Syst Bacteriol 1995a; 45: 429-435.

Frandsen EV, Reinholdt J, Kjeldsen M, Kilian M. *In vivo* cleavage of immunoglobulin A1 by immunoglobulin A1 proteases from *Prevotella* and *Capnocytophaga* species. Oral Microbiol Immunol 1995b; 10: 291-296.

Fujise O, Chen W, Rich S, Chen C. Clonal diversity and stability of subgingival *Eikenella corrodens*. J Clin Microbiol 2004; 42: 2036-2042.

Fukui K, Kato N, Kato H, Watanabe K, Tatematsu N. Incidence of *Prevotella intermedia* and *Prevotella nigrescens* carriage among family members with subclinical periodontal disease. J Clin Microbiol 1999; 37: 3141-3145.

Fukushima H, Moroi H, Inoue J, Onoe T, Ezaki T, Yabuuchi E, Leung KP, Walker CB, Clark WB, Sagawa H. Phenotypic characteristics and DNA relatedness in *Prevotella intermedia* and similar organisms. Oral Microbiol Immunol 1992; 7: 60-64.

Gaetti-Jardim EJ, Avila-Campos MJ. Haemagglutination and haemolysis by oral *Fusobacterium nucleatum*. New Microbiol 1999; 22: 63-67.

García L, Tercero JC, Legido B, Ramos JA, Alemany J, Sanz M. Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis* by multiplex PCR. J Periodont Res 1998; 33: 59-64.

George KS, Reynolds MA, Falkler WA. Arbitrarily primed polymerase chain reaction fingerprinting and clonal analysis of oral *Fusobacterium nucleatum* isolates. Oral Microbiol Immunol 1997; 12: 219-226.

Gharbia SE, Shah HN, Lawson PA, Haapasalo M. Distribution and frequency of *Fusobacterium nucleatum* subspecies in the human oral cavity. Oral Microbiol Immunol 1990; 5: 324-327.

Gharbia SE, Shah HN. *Fusobacterium nucleatum* subsp. *fusiforme* subsp. nov. and *Fusobacterium nucleatum* subsp. *animalis* subsp. nov. as additional subspecies within *Fusobacterium nucleatum*. Int J Syst Bacteriol 1992; 42: 296-298.

Gharbia SE, Haapasalo M, Shah HN, Kotiranta A, Lounatmaa K, Pearce MA, Devine DA. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. J Periodontol 1994; 65: 56-61.

Gibbons RJ. Adherent interactions which may affect microbial ecology in the mouth. J Dent Res 1984; 63: 378-385.

Gilbert P, Allison DG. Biofilms and their resistance towards antimicrobial agents. In: Dental plaque revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 125-143.

Gmür R, Thurnheer T. Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical specimens. Microbiology 2002; 148: 1379-1387.

Gmür R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. Gingival crevice microbiota from Chinese patients with gingivitis or necrotizing ulcerative gingivitis. Eur J Oral Sci 2004; 112: 33-41.

Goldhar J. Bacterial lectinlike adhesins: determination and specificity. Methods Enzymol 1994; 236: 211-231.

Goldhar J. Erythrocytes as target cells for testing bacterial adhesins. Methods Enzymol 1995; 253: 43-50.

Gonçalves RB, Robitaille M, Mouton C. Identical clonal types of *Porphyromonas gingivalis* or *Prevotella nigrescens* recovered from infected root canals and subgingival plaque. Oral Microbiol Immunol 1999; 14: 197-200.

Griffen AL, Becker MR, Lyons SR, Moeschberger ML, Leys EJ. Prevalence of *Porphyromonas gingivalis* and periodontal health status. J Clin Microbiol 1998; 36: 3239-3242.

Grönroos L, Alaluusua S. Site-specific oral colonization of mutans streptococci detected by arbitrarily primed PCR fingerprinting. Caries Res 2000; 34: 474-480.

Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994; 5: 78-111.

Haffajee AD, Socransky SS, Feres M, Ximenez-Fyvie LA. Plaque microbiology in health and disease. In: Dental plaque revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 255-282.

Hafström C, Dahlén G. Pathogenicity of *Prevotella intermedia* and *Prevotella nigrescens* isolates in a wound chamber model in rabbits. Oral Microbiol Immunol 1997; 12: 148-154.

Han YW, Shi W, Huang GT, Kinder Haake S, Park NH, Kuramitsu H, Genco RJ. Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. Infect Immun 2000; 68: 3140-3146.

Handal T, Olsen I. Antimicrobial resistance with focus on oral beta-lactamases. Eur J Oral Sci 2000; 108: 163-174.

Handley PS, McNab R, Jenkinson HF. Adhesive surface structures on oral bacteria. In: Dental plaque revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 145-170.

Hannula J, Saarela M, Jousimies-Somer H, Takala A, Syrjänen R, Könönen E, Asikainen S. Age-related acquisition of oral and nasopharyngeal yeast species and stability of colonization in young children. Oral Microbiol Immunol 1999; 14: 176-182.

Hartroth B, Seyfahrt I, Conrads G. Sampling of periodontal pathogens by paper points: evaluation of basic parameters. Oral Microbiol Immunol 1999; 14: 326-330.

Hirasawa M, Takada K. *Porphyromonas gingivicanis* sp. nov. and *Porphyromonas crevioricanis* sp. nov., isolated from beagles. Int J Syst Bacteriol 1994; 44: 637-640.

Hofstad T. The genus *Fusobacterium*. In: The Prokaryotes: an evolving electronic resource for the microbiological community. Dworkin Mea (ed). 1999; New York: Springer-Verlag.

Hohwy J, Kilian M. Clonal diversity of the *Streptococcus mitis* biovar 1 population in the human oral cavity and pharynx. Oral Microbiol Immunol 1995; 10: 19-25.

Hohwy J, Reinholdt J, Kilian M. Population dynamics of *Streptococcus mitis* in its natural habitat. Infect Immun 2001; 69: 6055-6063.

Holbrook WP, Duerden BI. A comparison of some characteristics of reference strains of *Bacteroides oralis* with *Bacteroides melaninogenicus*. Arch Oral Biol 1974; 19: 1231-1235.

Holbrook WP, Óskarsdóttir Á, Fridjónsson T, Einarsson H, Hauksson A, Geirsson RT. No link between low-grade periodontal disease and preterm birth: a pilot study in a healthy Caucasian population. Acta Odontol Scand 2004; 62: 177-179.

Holdeman LV, Johnson JL. Description of *Bacteroides loescheii* sp. nov. and emendation of the description of *Bacteroides melaninogenicus* (Oliver and Wherry) Roy and Kelly 1939 and *Bacteroides denticola* Shah and Collins 1981. Int J Syst Bacteriol 1982; 32: 399-409.

Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000 1999; 20: 168-238.

Hoshi M, Kato I, Goto N, Hasegawa K. Hemolytic toxin produced by *Porphyromonas gingivalis*. FEMS Microbiol Lett 1993; 114: 273-277.

Hossain H, Ansari F, Schulz-Weidner N, Wetzel WE, Chakraborty T, Domann E. Clonal identity of *Candida albicans* in the oral cavity and the gastrointestinal tract of pre-school children. Oral Microbiol Immunol 2003; 18: 302-308.

Jalava J, Eerola E. Phylogenetic analysis of *Fusobacterium alocis* and *Fusobacterium sulci* based on 16S rRNA gene sequences: proposal of *Filifactor alocis* (Cato, Moore and Moore) comb. nov. and *Eubacterium sulci* (Cato, Moore and Moore) comb. nov. Int J Syst Bacteriol 1999; 49: 1375-1379.

Jang J, Kim B, Lee J, Han H. A rapid method for identification of typical *Leuconostoc* species by 16S rDNA PCR-RFLP analysis. J Microbiol Methods 2003; 55: 295-302.

Jansen HJ, Grenier D, Van der Hoeven JS. Characterization of immunoglobulin G-degrading proteases of *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1995; 10: 138-145.

Jaulhac B, Reyrolle M, Sodahlon YK, Jarraud S, Kubina M, Monteil H, Piémont Y, Etienne J. Comparison of sample preparation methods for detection of *Legionella pneumophila* in culture-positive bronchoalveolar lavage fluids by PCR. J Clin Microbiol 1998; 36: 2120-2122.

Jeffcoat MK, Geurs NC, Reddy MS, Goldenberg RL, Hauth JC. Current evidence regarding periodontal disease as a risk factor in preterm birth. Ann Periodontol 2001; 6: 183-188.

Jensen S, Bergh Ø, Enger Ø, Hjeltnes B. Use of PCR-RFLP for genotyping 16S rRNA and characterizing bacteria cultured from halibut fry. Can J Microbiol 2002; 48: 379-386.

Jousimies-Somer H, Summanen P, Citron DM, Baron EJ, Wexler HM, Finegold SM. Wadsworth-KTL anaerobic bacteriology manual. 6th ed. 2002; Los Angeles: Star Publishing Company.

Kamma JJ, Diamanti-Kipioti A, Nakou M, Mitsis FJ. Profile of subgingival microbiota in children with mixed dentition. Oral Microbiol Immunol 2000; 15: 103-111.

Kaplan JB, Schreiner HC, Furgang D, Fine DH. Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients. J Clin Microbiol 2002; 40: 1181-1187.

Karunakaran T, Holt SC. Cloning of two distinct hemolysin genes from *Porphyromonas* (*Bacteroides*) *gingivalis* in *Escherichia coli*. Microb Pathog 1993; 15: 37-49.

Kelstrup J. The incidence of *Bacteroides melaninogenicus* in human gingival sulci, and its prevalence in the oral cavity at different ages. Periodontics 1966; 4: 14-18.

Kilian M. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect Immun 1981; 34: 757-765.

Kilian M, Reinholdt J, Lomholt H, Poulsen K, Frandsen EV. Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. APMIS 1996; 104: 321-338.

Klein JO. The burden of otitis media. Vaccine 2000; 19 (Suppl 1): S2-8.

Kolenbrander PE, Andersen RN, Moore LV. Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. Infect Immun 1989; 57: 3194-3203.

Kolenbrander PE, Andersen RN, Clemans DL, Whittaker CR, Klier CM. Potential role of functionally similar coaggregation mediators in bacterial succession. In: Dental plaque

revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 171-186.

Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000; 54: 413-437.

Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. J Periodont Res 1980; 15: 111-122.

Kuboniwa M, Amano A, Kimura KR, Sekine S, Kato S, Yamamoto Y, Okahashi N, Iida T, Shizukuishi S. Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes. Oral Microbiol Immunol 2004; 19: 168-176.

Kuhnert P, Frey J, Lang NP, Mayfield L. Phylogenetic analysis of *Prevotella nigrescens*, *Prevotella intermedia* and *Porphyromonas gingivalis* clinical strains reveals a clear species clustering. Int J Syst Evol Microbiol 2002; 52: 1391-1395.

Külekci G, Ciftci S, Keskin F, Kilic AO, Türkoglu S, Badur S, Develioglu ÖN, Leblebicioglu B, Külekci M. PCR analysis of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Fusorbacterium nucleatum* in middle ear effusion. Anaerobe 2001; 7: 241-246.

Köhler B, Bratthall D. Intrafamilial levels of *Streptococcus mutans* and some aspects of the bacterial transmission. Scand J Dent Res 1978; 86: 35-42.

Köhler B, Lundberg AB, Birkhed D, Papapanou PN. Longitudinal study of intrafamilial mutans streptococci ribotypes. Eur J Oral Sci 2003; 111: 383-389.

Könönen E, Asikainen S, Jousimies-Somer H. The early colonization of gram-negative anaerobic bacteria in edentulous infants. Oral Microbiol Immunol 1992; 7: 28-31.

Könönen E. Pigmented *Prevotella* species in the periodontally healthy oral cavity. FEMS Immunol Med Microbiol 1993; 6: 201-205.

Könönen E, Asikainen S, Saarela M, Karjalainen J, Jousimies-Somer H. The oral gram-negative anaerobic microflora in young children: longitudinal changes from edentulous to dentate mouth. Oral Microbiol Immunol 1994a; 9: 136-141.

Könönen E, Jousimies-Somer H, Asikainen S. The most frequently isolated gram-negative anaerobes in saliva and subgingival samples taken from young women. Oral Microbiol Immunol 1994b; 9: 126-128.

Könönen E, Saarela M, Karjalainen J, Jousimies-Somer H, Alaluusua S, Asikainen S. Transmission of oral *Prevotella melaninogenica* between a mother and her young child. Oral Microbiol Immunol 1994c; 9: 310-314.

Könönen E, Saarela M, Kanervo A, Karjalainen J, Asikainen S, Jousimies-Somer H. ß-Lactamase production and penicillin susceptibility among different ribotypes of *Prevotella melaninogenica* simultaneously colonizing the oral cavity. Clin Infect Dis 1995; 20 (Suppl 2): S364-366.

Könönen E, Nyfors S, Mättö J, Asikainen S, Jousimies-Somer H. ß-lactamase production by oral pigmented *Prevotella* species isolated from young children. Clin Infect Dis 1997; 25 (Suppl 2): S272-274.

Könönen E, Eerola E, Frandsen EV, Jalava J, Mättö J, Salmenlinna S, Jousimies-Somer H. Phylogenetic characterization and proposal of a new pigmented species to the genus *Prevotella*: *Prevotella pallens* sp. nov. Int J Syst Bacteriol 1998a; 48: 47-51.

Könönen E, Mättö J, Vaisanen-Tunkelrott ML, Frandsen EV, Helander I, Asikainen S, Finegold SM, Jousimies-Somer H. Biochemical and genetic characterization of a *Prevotella intermedia/nigrescens*-like organism. Int J Syst Bacteriol 1998b; 48: 39-46.

Könönen E. Oral colonization by anaerobic bacteria during childhood: role in health and disease. Oral Dis 1999; 5: 278-285.

Könönen E, Kanervo A, Bryk A, Takala A, Syrjänen R, Jousimies-Somer H. Anaerobes in the nasopharynx during acute otitis media episodes in infancy. Anaerobe 1999a; 5: 237-239.

Könönen E, Kanervo A, Salminen K, Jousimies-Somer H. β-Lactamase production and antimicrobial susceptibility of oral heterogeneous *Fusobacterium nucleatum* populations in young children. Antimicrob Agents Chemother 1999b; 43: 1270-1273.

Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. J Dent Res 1999c; 78: 1634-1639.

Könönen E. Development of oral bacterial flora in young children. Ann Med 2000; 32: 107-112.

Könönen E, Wolf J, Mättö J, Frandsen EV, Poulsen K, Jousimies-Somer H, Asikainen S. The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. J Periodont Res 2000; 35: 329-334.

Könönen E, Jousimies-Somer H, Bryk A, Kilpi T, Kilian M. Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age. J Med Microbiol 2002; 51: 723-730.

Könönen E, Syrjänen R, Takala A, Jousimies-Somer H. Nasopharyngeal carriage of anaerobes during health and acute otitis media by two years of age. Diagn Microbiol Infect Dis 2003; 46: 167-172.

Lacroix JM, Walker CB. Detection and prevalence of the tetracycline resistance determinant Tet Q in the microbiota associated with adult periodontitis. Oral Microbiol Immunol 1996; 11: 282-288.

Laguerre G, Mavingui P, Allard MR, Charnay MP, Louvrier P, Mazurier SI, Rigottier-Gois L, Amarger N. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. Appl Environ Microbiol 1996; 62: 2029-2036.

Latva-aho M, Pajukanta R, Sorsa T, Haraldsson G, Könönen E. Effect of Pregnancy on Periodontal Health Status. J Dent Res 2004; 84: Abstract 1104.

Lawson PA, Gharbia SE, Shah HN, Clark DR. Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. FEMS Microbiol Lett 1989; 53: 41-45.

Leung K, Nesbitt WE, Okamoto M, Fukushima H. Identification of a fimbriae-associated haemagglutinin from *Prevotella intermedia*. Microb Pathog 1999; 26: 139-148.

Leung KP, Fukushima H, Sagawa H, Walker CB, Clark WB. Surface appendages, hemagglutination, and adherence to human epithelial cells of *Bacteroides intermedius*. Oral Microbiol Immunol 1989; 4: 204-210.

Leung KP, Fukushima H, Nesbitt WE, Clark WB. *Prevotella intermedia* fimbriae mediate hemagglutination. Oral Microbiol Immunol 1996; 11: 42-50.

Lépine G, Progulske-Fox A. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. Oral Microbiol Immunol 1996; 11: 65-78.

Li Y, Caufield PW. Arbitrarily primed polymerase chain reaction fingerprinting for the genotypic identification of mutans streptococci from humans. Oral Microbiol Immunol 1998; 13: 17-22.

Li Y, Caufield PW, Emanuelsson IR, Thornqvist E. Differentiation of *Streptococcus mutans* and *Streptococcus sobrinus* via genotypic and phenotypic profiles from three different populations. Oral Microbiol Immunol 2001; 16: 16-23.

Loesche WJ. Ecology of the oral flora. In: Oral microbiology and immunology. Newman MG, Nisengard R (eds). 1988; Philadelphia: W. B. Saunders company, 351-366.

Loesche WJ. Association of the oral flora with important medical diseases. Curr Opin Periodontol 1997; 4: 21-28.

Loesche WJ, Lopatin DE. Interactions between periodontal disease, medical diseases and immunity in the older individual. Periodontol 2000 1998; 16: 80-105.

Logan NA. Bacterial systematics. 1994; Oxford: Blackwell Scientific Publications.

Loos BG, Mayrand D, Genco RJ, Dickinson DP. Genetic heterogeneity of *Porphyromonas* (*Bacteroides*) *gingivalis* by genomic DNA fingerprinting. J Dent Res 1990; 69: 1488-1493.

Love DN, Bailey GD, Collings S, Briscoe DA. Description of *Porphyromonas circumdentaria* sp. nov. and reassignment of *Bacteroides salivosus* (Love, Johnson, Jones, and Calverley 1987) as *Porphyromonas* (Shah and Collins 1988) *salivosa* comb. nov. Int J Syst Bacteriol 1992; 42: 434-438.

Love DN, Karjalainen J, Kanervo A, Forsblom B, Sarkiala E, Bailey GD, Wigney DI, Jousimies-Somer H. *Porphyromonas canoris* sp. nov., an asaccharolytic, black-pigmented species from the gingival sulcus of dogs. Int J Syst Bacteriol 1994; 44: 204-208.

Love DN. *Porphyromonas macacae* comb. nov., a consequence of *Bacteroides macacae* being a seenior synonym of *Porphyromonas salivosa*. Int J Syst Bacteriol 1995; 45: 90-92.

Madianos PN, Papapanou PN, Socransky SS, Dahlén G, Sandros J. Host-related genotypic heterogeneity of *Porphyromonas gingivalis* strains in the beagle dog. Oral Microbiol Immunol 1994; 9: 241-247.

Madianos PN, Papapanou PN, Nannmark U, Dahlén G, Sandros J. *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells in vitro. Infect Immun 1996; 64: 660-664.

Maidak BL, Cole JR, Lilburn TG, Parker CT, Jr., Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM. The RDP-II (Ribosomal Database Project). Nucleic Acids Res 2001; 29: 173-174.

Manch-Citron JN, Allen J, Moos M, Jr., London J. The gene encoding a *Prevotella loescheii* lectin-like adhesin contains an interrupted sequence which causes a frameshift. J Bacteriol 1992; 174: 7328-7336.

Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev 1998; 62: 71-109.

Marsh P, Martin M. Oral microbiology. 3th ed. 1992; London: Chapman & Hall.

Martin FE, Nadkarni MA, Jacques NA, Hunter N. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J Clin Microbiol 2002; 40: 1698-1704.

Mathis A, Weber R, Kuster H, Speich R. Simplified sample processing combined with a sensitive one-tube nested PCR assay for detection of *Pneumocystis carinii* in respiratory specimens. J Clin Microbiol 1997; 35: 1691-1695.

Mattos-Graner RO, Li Y, Caufield PW, Duncan M, Smith DJ. Genotypic diversity of mutans streptococci in Brazilian nursery children suggests horizontal transmission. J Clin Microbiol 2001; 39: 2313-2316.

Mättö J, Saarela M, von Troil-Lindén B, Alaluusua S, Jousimies-Somer H, Asikainen S. Similarity of salivary and subgingival *Prevotella intermedia* and *Prevotella nigrescens* isolates by arbitrarily primed polymerase chain reaction. Oral Microbiol Immunol 1996a; 11: 395-401.

Mättö J, Saarela M, von Troil-Lindén B, Könönen E, Jousimies-Somer H, Torkko H, Alaluusua S, Asikainen S. Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1996b; 11: 96-102.

Mättö J, Asikainen S, Vaisanen ML, Rautio M, Saarela M, Summanen P, Finegold S, Jousimies-Somer H. Role of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* in extraoral and some odontogenic infections. Clin Infect Dis 1997; 25 (Suppl 2): S194-198.

Mättö J, Saarela M, Alaluusua S, Oja V, Jousimies-Somer H, Asikainen S. Detection of *Porphyromonas gingivalis* from saliva by PCR by using a simple sample-processing method. J Clin Microbiol 1998; 36: 157-160.

Mättö J, Asikainen S, Vaisanen ML, Von Troil-Linden B, Könönen E, Saarela M, Salminen K, Finegold SM, Jousimies-Somer H. ß-lactamase production in *Prevotella intermedia*, *Prevotella nigrescens*, and *Prevotella pallens* genotypes and in vitro susceptibilities to selected antimicrobial agents. Antimicrob Agents Chemother 1999; 43: 2383-2388.

Meunier JR, Grimont PA. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Res Microbiol 1993; 144: 373-379.

Ménard C, Mouton C. Clonal diversity of the taxon *Porphyromonas gingivalis* assessed by random amplified polymorphic DNA fingerprinting. Infect Immun 1995; 63: 2522-2531.

Milsom SE, Sprague SV, Dymock D, Weightman AJ, Wade WG. Rapid differentiation of *Prevotella intermedia* and *P. nigrescens* by 16S rDNA PCR-RFLP. J Med Microbiol 1996; 44: 41-43.

Moore LV, Johnson JL, Moore WE. Descriptions of *Prevotella tannerae* sp. nov. and *Prevotella enoeca* sp. nov. from the human gingival crevice and emendation of the description of *Prevotella zoogleoformans*. Int J Syst Bacteriol 1994; 44: 599-602.

Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Palcanis KG, Ranney RR. Comparative bacteriology of juvenile periodontitis. Infect Immun 1985; 48: 507-519.

Moore WEC, Moore LVH. The bacteria of periodontal diseases. Periodontol 2000 1994; 5: 66-77.

Moraes SR, Siqueira JF, Jr., Rocas IN, Ferreira MC, Domingues RM. Clonality of *Fusobacterium nucleatum* in root canal infections. Oral Microbiol Immunol 2002; 17: 394-396.

Morillo JM, Lau L, Sanz M, Herrera D, Silva A. Quantitative real-time PCR based on single copy gene sequence for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Periodontal Res 2003; 38: 518-524.

Morris ML, Andrews RH, Rogers AH. Investigations of the taxonomy and systematics of *Fusobacterium nucleatum* using allozyme electrophoresis. Int J Syst Bacteriol 1997; 47: 103-110.

Moyer CL, Tiedje JM, Dobbs FC, Karl DM. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl Environ Microbiol 1996; 62: 2501-2507.

Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002; 81: 761-766.

Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 2004; 42: 3023-3029.

Möhlenhoff P, Müller L, Gorbushina AA, Petersen K. Molecular approach to the characterisation of fungal communities: methods for DNA extraction, PCR amplification and DGGE analysis of painted art objects. FEMS Microbiol Lett 2001; 195: 169-173.

Möller ÅJ. Microbiological examination of root canals and periapical tissues of human teeth. 1966; Göteborg: Akademiförlaget.

Nadkarni MA, Caldon CE, Chhour K-L, Fisher IP, Martin FE, Jacques NA, Hunter N. Carious dentine provides a habitat for a complex array of novel *Prevotella*-like bacteria. J Clin Microbiol 2004; 42: 5238-5244.

Narongwanichgarn W, Kawaguchi E, Misawa N, Goto Y, Haga T, Shinjo T. Differentiation of *Fusobacterium necrophorum* subspecies from bovine pathological lesions by RAPD-PCR. Vet Microbiol 2001; 82: 383-388.

Nishikata M, Yoshimura F, Nodasaka Y. Possibility of *Bacteroides gingivalis* hemagglutinin possessing protease activity revealed by inhibition studies. Microbiol Immunol 1989; 33: 75-80.

Nishikata M, Yoshimura F. Characterization of *Porphyromonas* (*Bacteroides*) *gingivalis* hemagglutinin as a protease. Biochem Biophys Res Commun 1991; 178: 336-342.

Nyfors S, Könönen E, Takala A, Jousimies-Somer H. β-Lactamase production by oral anaerobic gram-negative species in infants in relation to previous antimicrobial therapy. Antimicrob Agents Chemother 1999; 43: 1591-1594.

Nyfors S, Könönen E, Syrjänen R, Komulainen E, Jousimies-Somer H. Emergence of penicillin resistance among *Fusobacterium nucleatum* populations of commensal oral flora during early childhood. J Antimicrob Chemother 2003; 51: 107-112.

Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, McKaig R, Beck J. Periodontal infection as a possible risk factor for preterm low birth weight. J Periodontol 1996; 67: 1103-1113.

Ogawa T, Hamada S. Hemagglutinating and chemotactic properties of synthetic peptide segments of fimbrial protein from *Porphyromonas gingivalis*. Infect Immun 1994; 62: 3305-3310.

Okamoto M, Maeda N, Kondo K, Leung KP. Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1999; 178: 299-304.

Olsen I, Shah HN. International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of Gram-negative anaerobic rods: Minutes of the meeting, 29 July 2002, Paris, France. Int J Syst Evol Microbiol 2003; 53: 923-924.

Olsvik B, Tenover FC. Tetracycline resistance in periodontal pathogens. Clin Infect Dis 1993; 16 (Suppl 4): S310-313.

Olsvik B, Flynn MJ, Tenover FC, Slots J, Olsen I. Tetracycline resistance in Prevotella isolates from periodontally diseased patients is due to the tet(Q) gene. Oral Microbiol Immunol 1996; 11: 304-308.

Ozaki M, Miyake Y, Shirakawa M, Takemoto T, Okamoto H, Suginaka H. Binding specificity of *Fusobacterium nucleatum* to human erythrocytes, polymorphonuclear leukocytes, fibroblasts, and HeLa cells. J Periodont Res 1990; 25: 129-134.

Paju S, Carlson P, Jousimies-Somer H, Asikainen S. Heterogeneity of *Actinobacillus actinomycetemcomitans* strains in various human infections and relationships between serotype, genotype, and antimicrobial susceptibility. J Clin Microbiol 2000; 38: 79-84.

Pan YP, Li Y, Caufield PW. Phenotypic and genotypic diversity of *Streptococcus sanguis* in infants. Oral Microbiol Immunol 2001; 16: 235-242.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. J Bacteriol 2001; 183: 3770-3783.

Pearce C, Bowden GH, Evans M, Fitzsimmons SP, Johnson J, Sheridan MJ, Wientzen R, Cole MF. Identification of pioneer viridans streptococci in the oral cavity of human neonates. J Med Microbiol 1995; 42: 67-72.

Premaraj T, Kato N, Fukui K, Kato H, Watanabe K. Use of PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis techniques for differentiation of *Prevotella intermedia* sensu stricto and *Prevotella nigrescens*. J Clin Microbiol 1999; 37: 1057-1061.

Quirynen M, De Soete M, Dierickx K, van Steenberghe D. The intra-oral translocation of periodontopathogens jeopardises the outcome of periodontal therapy. A review of the literature. J Clin Periodontol 2001; 28: 499-507.

Redmo Emanuelsson IM, Wang XM. Demonstration of identical strains of mutans streptococci within Chinese families by genotyping. Eur J Oral Sci 1998; 106: 788-794.

Redmo Emanuelsson IM, Thornqvist E. Genotypes of mutans streptococci tend to persist in their host for several years. Caries Res 2000; 34: 133-139.

Redmo Emanuelsson IM, Carlsson P, Hamberg K, Bratthall D. Tracing genotypes of mutans streptococci on tooth sites by random amplified polymorphic DNA (RAPD) analysis. Oral Microbiol Immunol 2003; 18: 24-29.

Riggio MP, Lennon A, Roy KM. Detection of *Prevotella intermedia* in subgingival plaque of adult periodontitis patients by polymerase chain reaction. J Periodont Res 1998; 33: 369-376.

Roques CG, El kaddouri S, Barthet P, Duffort JF, Arellano M. *Fusobacterium nucleatum* involvement in adult periodontitis and possible modification of strain classification. J Periodontol 2000; 71: 1144-1150.

Rotimi VO, Duerden BI. The development of the bacterial flora in normal neonates. J Med Microbiol 1981; 14: 51-62.

Ruby JD, Li Y, Luo Y, Caufield PW. Genetic characterization of the oral *Actinomyces*. Arch Oral Biol 2002; 47: 457-463.

Ruiz A, Poblet M, Mas A, Guillamón JM. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int J Syst Evol Microbiol 2000; 50 Pt 6: 1981-1987.

Saarela M, Stucki AM, von Troil-Lindén B, Alaluusua S, Jousimies-Somer H, Asikainen S. Intra- and inter-individual comparison of *Porphyromonas gingivalis* genotypes. FEMS Immunol Med Microbiol 1993a; 6: 99-102.

Saarela M, von Troil-Lindén B, Torkko H, Stucki AM, Alaluusua S, Jousimies-Somer H, Asikainen S. Transmission of oral bacterial species between spouses. Oral Microbiol Immunol 1993b; 8: 349-354.

Saarela MH, Dogan B, Alaluusua S, Asikainen S. Persistence of oral colonization by the same *Actinobacillus actinomycetemcomitans* strain(s). J Periodontol 1999; 70: 504-509.

Sakamoto M, Suzuki M, Huang Y, Umeda M, Ishikawa I, Benno Y. *Prevotella shahii* sp. nov. and *Prevotella salivae* sp. nov., isolated from the human oral cavity. Int J Syst Evol Microbiol 2004; 54: 877-883.

Sakamoto M, Huang Y, Umeda M, Ishikawa I, Benno Y. *Prevotella multiformis* sp. nov., isolated from human subgingival plaque. Int J Syst Evol Microbiol 2005; 55: 815-819.

Sandros J, Papapanou P, Dahlén G. *Porphyromonas gingivalis* invades oral epithelial cells in vitro. J Periodontal Res 1993; 28: 219-226.

Scannapieco FA. Role of oral bacteria in respiratory infection. J Periodontol 1999; 70: 793-802.

Schleifer KHL, W. Molecular taxonomy: Classification and identification. In: Bacterial Diversity and Systematics. Priest FG, Ramos-Cormenzana A, Tindall BJ (eds). 1994; New York: Plenum Press, 1-15.

Shah HN, Collins MD. Proposal for reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis,* and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int J Syst Bacteriol 1988; 38: 128-131.

Shah HN, Collins MD. Proposal to restrict the genus *Bacteroides* (Castellani and Chalmers) to *Bacteroides fragilis* and closely related species. Int J Syst Bacteriol 1989; 39: 85-87.

Shah HN, Gharbia SE. Lysis of erythrocytes by the secreted cysteine proteinase of *Porphyromonas gingivalis* W83. FEMS Microbiol Lett 1989; 61: 213-217.

Shah HN, Collins DM. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Bacteriol 1990; 40: 205-208.

Shah HN, Gharbia SE. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. Int J Syst Bacteriol 1992; 42: 542-546.

Shah HN, Gharbia SE, Progulske-Fox A, Brocklehurst K. Evidence for independent molecular identity and functional interaction of the haemagglutinin and cysteine proteinase (gingivain) of *Porphyromonas gingivalis*. J Med Microbiol 1992; 36: 239-244.

Shah HN, Collins MD, Olsen I, Paster BJ, Dewhirst FE. Reclassification of *Bacteroides levii* (Holdeman, Cato and Moore) in the genus *Porphyromonas* as *Porphyromonas levii* comb. nov. Int J Syst Bacteriol 1995a; 45: 586-588.

Shah HN, Gharbia SE, Scully C, Finegold SM. Oligonucleotide probes to the 16S ribosomal RNA: implications of sequence homology and secondary structure with particular reference to the oral species *Prevotella intermedia* and *Prevotella nigrescens*. Oral Dis 1995b; 1: 32-36.

Sheikhi M, Gustafsson A, Jarstrand C. Cytokine, elastase and oxygen radical release by *Fusobacterium nucleatum*-activated leukocytes: a possible pathogenic factor in periodontitis. J Clin Periodontol 2000; 27: 758-762.

Shinzato T, Saito A. A mechanism of pathogenicity of "*Streptococcus milleri* group" in pulmonary infection: synergy with an anaerobe. J Med Microbiol 1994; 40: 118-123.

Skår CK, Krüger PG, Bakken V. Characterisation and subcellular localisation of the GroEL-like and DnaK-like proteins isolated from *Fusobacterium nucleatum* ATCC 10953. Anaerobe 2003; 9: 305-312.

Slots J, Genco RJ. Direct hemagglutination technique for differentiating *Bacteroides asaccharolyticus* oral strains from nonoral strains. J Clin Microbiol 1979; 10: 371-373.

Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984; 63: 412-421.

Slots J, Ashimoto A, Flynn MJ, Li G, Chen C. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. Clin Infect Dis 1995; 20 (Suppl 2): S304-307.

Slots J. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease: introduction. Periodontol 2000 1999; 20: 7-13.

Smith DJ, King WF, Gilbert JV, Taubman MA. Structural integrity of infant salivary immunoglobulin A (IgA) in IgA1 protease-rich environments. Oral Microbiol Immunol 1998; 13: 89-96.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998; 25: 134-144.

Spratt DA, Weightman AJ, Wade WG. Diversity of oral asaccharolytic *Eubacterium* species in periodontitis--identification of novel phylotypes representing uncultivated taxa. Oral Microbiol Immunol 1999; 14: 56-59.

Stubbs S, Park SF, Bishop PA, Lewis MA. Direct detection of *Prevotella intermedia* and *P. nigrescens* in suppurative oral infection by amplification of 16S rRNA gene. J Med Microbiol 1999; 48: 1017-1022.

Suchett-Kaye G, Décoret D, Barsotti O. Clonal analysis by ribotyping of *Fusobacterium nucleatum* isolates obtained from healthy young adults with optimal plaque control. J Periodont Res 1998; 33: 179-186.

Suchett-Kaye G, Décoret D, Barsotti O. Intra-familial distribution of *Fusobacterium nucleatum* strains in healthy families with optimal plaque control. J Clin Periodontol 1999; 26: 401-404.

Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. Appl Microbiol 1972; 24: 638-644.

Syrjänen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. J Infect Dis 2001; 184: 451-459.

Takada K, Fukatsu A, Otake S, Hirasawa M. Isolation and characterization of hemolysin activated by reductant from *Prevotella intermedia*. FEMS Immunol Med Microbiol 2003; 35: 43-47.

Tancrède C. Role of human microflora in health and disease. Eur J Clin Microbiol Infect Dis 1992; 11: 1012-1015.

Tanner A, Lai C-H, Maiden M. Characteristics of oral Gram-negative species. In: Contemporary Oral Microbiology and Immunology. Slots J, Taubman MA (eds). 1992; St. Louis: Mosby Year Book, 299-341.

Tanner A, Maiden MFJ, Paster BJ, Dewhirst FE. The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol 2000 1994; 5: 26-51.

Tanner AC, Goodson JM. Sampling of microorganisms associated with periodontal disease. Oral Microbiol Immunol 1986; 1: 15-22.

Teanpaisan R, Douglas CW, Walsh TF. Characterisation of black-pigmented anaerobes isolated from diseased and healthy periodontal sites. J Periodont Res 1995; 30: 245-251.

Teanpaisan R, Douglas CW, Eley AR, Walsh TF. Clonality of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontally diseased and healthy sites. J Periodont Res 1996; 31: 423-432.

Thurnheer T, Guggenheim B, Gruica B, Gmür R. Infinite serovar and ribotype heterogeneity among oral *Fusobacterium nucleatum* strains? Anaerobe 1999; 5: 79-92.

Tuttle RS, Strubel NA, Mourad J, Mangan DF. A non-lectin-like mechanism by which Fusobacterium nucleatum 10953 adheres to and activates human lymphocytes. Oral Microbiol Immunol 1992; 7: 78-83.

Tyler KD, Wang G, Tyler SD, Johnson WM. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. J Clin Microbiol 1997; 35: 339-346.

Urakawa H, Kita-Tsukamoto K, Ohwada K. 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family Vibrionaceae. FEMS Microbiol Lett 1997; 152: 125-132.

van Steenbergen TJ, Menard C, Tijhof CJ, Mouton C, de Graaff J. Comparison of three molecular typing methods in studies of transmission of *Porphyromonas gingivalis*. J Med Microbiol 1993a; 39: 416-421.

van Steenbergen TJ, Petit MD, Scholte LH, van der Velden U, de Graaff J. Transmission of *Porphyromonas gingivalis* between spouses. J Clin Periodontol 1993b; 20: 340-345.

van Steenbergen TJ, Bosch-Tijhof CJ, Petit MD, van der Velden U. Intra-familial transmission and distribution of *Prevotella intermedia* and *Prevotella nigrescens*. J Periodont Res 1997; 32: 345-350.

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 1996; 60: 407-438.

von Troil-Lindén B, Saarela M, Mättö J, Alaluusua S, Jousimies-Somer H, Asikainen S. Source of suspected periodontal pathogens re-emerging after periodontal treatment. J Clin Periodontol 1996; 23: 601-607.

Wade W. Unculturable bacteria in oral biofilms. In: Dental plaque revisited-oral biofilms in health and disease. Newman HN, Wilson M (eds). 1999; Cardiff: BioLine, 313-322.

Wade WG, Spratt DA, Dymock D, Weightman AJ. Molecular detection of novel anaerobic species in dentoalveolar abscesses. Clin Infect Dis 1997; 25 (Suppl 2): S235-236.

Walker CB, Bueno LC. Antibiotic resistance in an oral isolate of *Prevotella intermedia*. Clin Infect Dis 1997; 25 (Suppl 2): S281-283.

Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 1991; 10: 506-513.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991; 173: 697-703.

Weiss EI, London J, Kolenbrander PE, Andersen RN. Fimbria-associated adhesin of *Bacteroides loescheii* that recognizes receptors on procaryotic and eucaryotic cells. Infect Immun 1989; 57: 2912-2913.

Willems A, Collins MD. 16S rRNA gene similarities indicate that *Hallella seregens* (Moore and Moore) and *Mitsuokella dentalis* (Haapasalo *et al*.) are genealogically highly related and are members of the genus *Prevotella*: emended description of the genus *Prevotella* (Shah and Collins) and description of *Prevotella dentalis* comb. nov. Int J Syst Bacteriol 1995a; 45: 832-836.

Willems A, Collins MD. Reclassification of *Oribaculum catoniae* (Moore and Moore 1994) as *Porphyromonas catoniae* comb. nov. and emendation of the genus *Porphyromonas*. Int J Syst Bacteriol 1995b; 45: 578-581.

Wilson G. The normal flora of man: Introduction, general considerations and importance. In: The normal flora of man. Skinner FA, Carr JG (eds). 1974; London: Academic Press, 1-5.

Wu CC, Johnson JL, Moore WE, Moore LV. Emended descriptions of *Prevotella denticola*, *Prevotella loescheii*, *Prevotella veroralis*, and *Prevotella melaninogenica*. Int J Syst Bacteriol 1992; 42: 536-541.

Xia T, Baumgartner JC, David LL. Isolation and identification of *Prevotella tannerae* from endodontic infections. Oral Microbiol Immunol 2000; 15: 273-275.

Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 2000; 27: 648-657.

Yoneda M, Kuramitsu HK. Genetic evidence for the relationship of *Porphyromonas gingivalis* cysteine protease and hemagglutinin activities. Oral Microbiol Immunol 1996; 11: 129-134.

Yoshida A, Tachibana M, Ansai T, Takehara T. Multiplex polymerase chain reaction assay for simultaneous detection of black-pigmented *Prevotella* species in oral specimens. Oral Microbiol Immunol 2005; 20: 43-46.

Yoshimura F, Takahashi K, Nodasaka Y, Suzuki T. Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J Bacteriol 1984; 160: 949-957.

Zambon JJ. Periodontal diseases: microbial factors. Ann Periodontol 1996; 1: 879-925.