Molecular Analysis of the Oral Microbiota of Dental Diseases

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As you set out for Ithaka
hope the voyage is a long one,
full of adventure, full of discovery.
Laistrygonians and Cyclops,
angry Poseidon - don’t be afraid of them:
you’ll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement stirs your spirit and your body.
...Keep Ithaka always in your mind.
Arriving there is what you are destined for...
And if you find her poor, Ithaka won’t have fooled you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.

ITHAKA, Constantine Petrou Cavafy, 1911
Translated by Edmund Keeley/Philip Sherrard

In memory of my grandfather Spyridona Iatridi
Dedicated to Irini Iatridou-Kanasi, Spyridona Kanasi,
Thoma Thomou
Abstract

Traditionally, bacterial culture has been used for bacterial detection, allowing study of living microorganisms. Molecular methods are rapid and allow simultaneous identification of numerous species and uncultivated phylotypes. The objective of this doctoral thesis was to investigate the role of the oral microbiota, including poorly characterized and uncultivated bacteria, in dental caries and periodontitis, by comprehensive molecular, clinical, and statistical methods. The microbiota of 275 preschool children (75 with caries and 200 caries-free) was examined by whole genomic DNA probes, 16S rDNA cloning and sequencing, and PCR. *Streptococcus mutans*, exhibiting a combined association with *Streptococcus sobrinus*, was significantly associated with Early Childhood Caries (ECC). Plaque from children with Severe Early Childhood Caries (S-ECC) was diverse with 138 identified and 107 unidentified taxa, which possibly included novel phylotypes. Other species/phylotypes associated with childhood caries included *Lactobacillus gasseri* (p<0.01), *Lactobacillus fermentum*, *Actinomyces israelii*, and *Actinomyces odontolyticus* (all p<0.05, ECC), *Veillonella parvula* (p<0.01), *Veillonella atypica* (p<0.05), and *Veillonella* sp. HOT-780 (p<0.01, S-ECC). *Lactobacillus acidophilus* and *Lactobacillus reuteri*, both used as probiotic therapy species, were detected more frequently in caries-free children than those with ECC. Fastidious periodontal species, including *Parvimonas micra*, *Aggregatibacter actinomycetemcomitans*, *Eubacterium brachy*, *Filifactor alocis* (all p<0.05), and *Porphyromonas gingivalis* (p<0.01), were also more frequently detected in children with dental caries than in caries-free children. Other variables associated with ECC were race, dental visit, snacking (all p<0.05), and visible dental plaque (p<0.01). The oral microbiota of early periodontitis in young adults (N=141) was analyzed by whole genomic and oligonucleotide DNA probes, and PCR. Species detected more frequently in early periodontitis than periodontal health included *Treponema denticola*, *F. alocis*, *Porphyromonas endodontalis*, *Bacteroides* sp. HOT-274 (oral clone AU126), and *A. odontolyticus* (p<0.01) by oligonucleotide DNA probes, and *P. gingivalis* (p<0.001) and *T. forsythia* (p=0.03) by PCR. Subgingival samples exhibited a higher prevalence of periodontitis-associated species than samples from tongue surface, including *A. actinomycetemcomitans*, *T. denticola*, *T. forsythia* (all p<0.05), and uncultivated TM7, *Treponema*, and *Actinobaculum* clones (all p<0.05). *P. gingivalis* (p<0.01) by PCR was associated with periodontal disease progression. Early periodontitis was associated with older age (p=0.01), male gender (p=0.04), and cigarette smoking (p=0.05). The role of bacterial subgroups in periodontitis was examined by studying the serotypeability of 313 genotyped clinical *A. actinomycetemcomitans* isolates (189 subjects). A total of 95 strains (30 subjects) remained non-serotypeable, although PCR revealed presence of the serotype-specific genes. The absence of the immunodominant serotype-specific antigen was confirmed by immunoblot assays. No major DNA rearrangement in the studied serotype-specific gene clusters was found. In summary, detection of previously cultured species and uncultivated phylotypes revealed the diversity of the oral microbiota in dental diseases and health already early in life. Bacterial species have insufficiently characterized subgroups that may have attributes to evade the host response. Molecular approaches used in this study enable comprehensive, culture-independent characterization of the oral microbiome that may in the future lead to identification of diagnostic bacterial profiles for dental diseases.

Key words: Early Childhood Caries, Early Periodontitis, 16S rDNA cloning and sequencing, whole genomic DNA probes, oligonucleotide DNA probes, PCR, diversity, molecular, *Aggregatibacter actinomycetemcomitans*, serotypes
# Table of Contents

List of Papers.............................................................................................................. 6

**Introduction**............................................................................................................... 7

- Genomic Era of Microbiological Identification......................................................... 7
- Oral Microbiota........................................................................................................ 8
- Dental Plaque – Colonization Sites and Diversity.................................................... 9

**Dental Diseases**...................................................................................................... 10

- Bacterial Etiology of Dental Diseases – Hypotheses............................................... 11
- Dental Caries – Caries Development and Risk Factors............................................. 11
- Dental Caries in Children....................................................................................... 12
- Microbiota of Dental Caries in Children.................................................................. 13
- Periodontal Diseases and Risk Factors.................................................................... 15
- Microbiota of Periodontal Health and Disease....................................................... 16

**Molecular Microbiological Identification of Oral Bacteria**.................................. 18

**Hypothesis and Aims**............................................................................................. 20

**Materials and Methods**......................................................................................... 21

- Study Populations and Clinical Measurements...................................................... 21
- Microbial Analysis................................................................................................ 23
- DNA-DNA Hybridization...................................................................................... 23
- Polymerase Chain Reaction.................................................................................. 24
- Cloning and Sequencing...................................................................................... 25
- Antigen Identification........................................................................................... 26
- Statistical Analysis................................................................................................ 26

**Results and Discussion**........................................................................................ 28

- Oral Microbiota in Patients with Childhood Caries................................................ 28
- Mutans and Non-mutans Streptococci................................................................. 30
- *Lactobacillus* Species.......................................................................................... 31
- Other Species........................................................................................................ 32
- Periodontitis-associated Bacteria.......................................................................... 34
- Socio-demographic, Dietary, and Clinical Factors.................................................. 34
- Oral Microbiota of Early Adult Periodontitis......................................................... 36
- Microbiota of Early Periodontitis and Periodontally Healthy Subjects.................. 36
- Subgingival and Tongue Surface Microbiota......................................................... 38
- Progressing Chronic Periodontitis........................................................................ 38
- Socio-demographic Factors in Early Periodontitis................................................ 40

*Aggregatibacter actinomycetemcomitans* Lack of Serotype Antigen Expression........ 40

**Summary and Conclusions**.................................................................................. 42

**Acknowledgments**............................................................................................... 44

**References**............................................................................................................. 46

**Papers I-V**............................................................................................................. Appendix I-V
List of Papers

The original papers that comprise this doctoral thesis are listed below and will be referred to by Roman numerals in the text.


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Introduction

Genomic Era of Microbiological Identification

Historically, bacteria have been isolated by culture methods and classified according to their phenotypic features, including cellular characteristics and metabolic profiles. Bacterial cultivation made it possible to work with living microorganisms, examine their physiology, antimicrobial resistance, and pathogenicity. In medical microbiology, commensal and pathogenic microorganisms were isolated in pure cultures using artificial media in well-defined conditions. This approach was fruitful and essential for defining the field of microbiology. Despite the extensive microbial cultivation, however, it has been estimated that >99% of microorganisms found in nature cannot be cultured using standard techniques (Hugenholtz et al., 1998). Moreover, researchers have been for years biased towards evaluations of single microorganisms in planktonic states, which rarely reflects the complexity of *in vivo* biofilms (Kolter, 2005).

In the 1970s, DNA-DNA hybridization was introduced for differentiating bacterial species, where homology greater than 70% between isolates defined the same species (Medini *et al.*, 2008). Ten years later, the introduction of the 16S rRNA macromolecule to facilitate determination of phylogenetic relationships (Lane *et al.*, 1985), marked a new genomic era for microbiologists. The 16S rRNA molecule was chosen for its universal distribution, high information content, large enough size (1,500 bp) for informatics purposes, and inclusion of highly conserved primer binding sites, as well as hypervariable regions which could provide species-specific sequences (Janda & Abbott, 2007). Following the improvement and availability of DNA techniques, the field of molecular microbiology has greatly expanded.

One of the major advances of molecular microbiology was recognizing the prokaryotic diversity. Novel phylotypes are continuously being identified from different environments. Multidisciplinary approaches, including statistical and mathematical models, are used to manage the vast amount of data produced (Medini *et al.*, 2008). The field of metagenomics has also been created to address whole community genomes and facilitate examination of the diversity of cultured and uncultivated microorganisms (Stein *et al.*, 1996). The extent of prokaryotic diversity
is a complicated issue which has divided the scientific community into two opposing sides; those who believe that the diversity is large (Hugenholtz et al., 1998) and those who claim that fewer novel sequences are obtained annually suggesting that most taxa have been identified (Hagstrom et al., 2002). Differences could be in part due to varying methodologies, differing microbial environments, and sample sizes (Curtis et al., 2006).

Organization complexity of microbial communities has also been a major recent research focus. Bacterial biofilms, matrix-enclosed populations of bacteria which adhere to surfaces and to each other, are diverse and exhibit intricate architectural characteristics, such as nutrient channels (Costerton et al., 1999). Molecular genetics are used in biofilm research to identify genes and regulatory pathways required for biofilm development (Davey & O'Toole G, 2000). Sessile bacterial cells forming biofilms differ from planktonic cells observed in suspended culture in terms of protein and genomic expression (Sauer & Camper, 2001; Sauer et al., 2002). Gene expression involved for instance in motility, intra- and inter-species communication, and antibiotic resistance, differs in bacteria growing as biofilms compared with those in planktonic lifeform (Davey & O'Toole G, 2000; Gjersing et al., 2007; Kolter & Greenberg, 2006; Whiteley et al., 2001). Understanding bacterial biofilm formation and regulation can be pivotal from a clinical viewpoint, since multiple chronic infections in the human body, such as osteomyelitis, endocarditis, cystic fibrosis pneumonia, otitis media, periodontitis, and dental caries, involve biofilms (Costerton et al., 1999).

**Oral Microbiota**

The human oral cavity is a diverse environment with hard and soft tissues comprising a total area of 215 cm² bathed in saliva (Collins & Dawes, 1987). The oral environment is thus optimal for microorganisms to grow as biofilms, similar in their architecture and characteristics to biofilms in nature (Auschill et al., 2001). Specific to oral biofilms is the ability of sessile oral bacterial cells to tolerate short-term abundance of external nutrient supply and to withstand nutrient restraint (Carlsson & Johansson, 1973). Nutrient deprived biofilm cells after reactivation have been shown to exhibit low reactivity in vitro, suggesting that slower reactivation of these cells might be a survival strategy (Chavez de Paz et al., 2008). Bacterial cells in oral
biofilms interact by various recognized ways including co-aggregation (Kolenbrander et al., 2006), metabolic exchange, cell-cell communication (Li et al., 2002), and exchange of genetic material (Roberts et al., 2001). These mechanisms benefit bacterial survival and can make dental biofilms difficult therapeutic targets in dental diseases.

**Dental Plaque – Colonization Sites and Diversity**

Oral biofilms are far from uniform in their composition. The healthy oral cavity was shown by culture to harbor mainly *Actinomyces, Streptococcus* species and *Rothia dentocariosa* (Moore et al., 1987). By 16S rDNA cloning and sequencing, the oral cavity of healthy subjects exhibited a specific bacterial flora according to the sample site and the individual, which was not associated with the dental disease microbiota (Aas et al., 2005).

Supragingival dental plaque has been extensively studied from initial colonization of bacteria on tooth surfaces to dental caries development. Initial colonizers in the dental biofilm seem to be dominated by *Streptococcus* species (Diaz et al., 2006; Dige et al., 2007; Kilian & Rolla, 1976; Socransky et al., 1977). Other “pioneering” genera observed are Gram-positive *Actinomyces, Gemella, Granulicatella,* and *Rothia* species, as well as Gram-negative *Neisseria, Prevotella,* and *Veillonella* species. Dental plaque on tooth surfaces from healthy volunteers consists mainly of Gram-positive organisms with coccoid cells (Listgarten, 1976). Epithelial cells seem to also be present in the initial biofilms (Kolenbrander et al., 2006; Loe et al., 1965). The degrading host cells could serve as nutrient depots for the bacteria (Diaz et al., 2006).

Subgingival plaque forms from apical migration of the plaque growing on the tooth surface (Theilade & Theilade, 1985). Histological sections of human subgingival plaque have revealed two zones of microorganisms. One zone of bacteria is clearly attached to the tooth surface and the other is lining the epithelial surface of the pocket (Listgarten, 1976; Socransky & Haffajee, 2002). Spirochetes and Gram-negative bacteria have been found to dominate the subgingival biofilm (Listgarten, 1976). Bacteria detected by immunohistochemical and DNA hybridization methods in the deeper sections of the epithelial surface biofilms include asaccharolytic
obligate anaerobes Porphyromonas gingivalis, Tannerella forsythia (previously Bacteroides forsythus, Tannerella forsythensis) and Treponema denticola species that are recognized periodontal pathogens (American Academy of Periodontology, 1996; Dibart et al., 1998; Kigure et al., 1995).

Bacterial analysis of soft tissue surfaces by whole genomic DNA probes, revealed marked differences in bacterial composition and proportions in the soft tissues sampled, and the tongue microbiota exhibited great similarity with bacteria in saliva (Mager et al., 2003). The tongue microbiota has been of special interest, since the tongue can harbor high biomasses of colonizing bacteria, including black-pigmented and other species able to produce volatile sulfur compounds associated with halitosis (Claesson et al., 1990; Persson et al., 1990; Spencer et al., 2007). It has also been proposed that the tongue could serve as a reservoir of pathogenic microorganisms and thus contribute to dental disease development (Faveri et al., 2006; Kazor et al., 2003; Tanner et al., 2002). Reports have indicated that halitosis could be an oral issue in both presence and absence of dental diseases, thus leaving the role of the tongue microbiota in dental disease development unsolved (Bosy et al., 1994; De Boever & Loesche, 1995; Hughes & McNab, 2008).

Dental Diseases

Bacterial infection is a necessary factor for the etiology of dental diseases. However, both dental caries and periodontal diseases (gingivitis and periodontitis) are multifactorial in their pathogenesis. Despite decades of scientific research and health education, they still affect a major portion of the population.

According to the World Health Organization (WHO), the prevalence of dental caries worldwide among adults is nearly 100% of the population [Decayed Missing Filled Teeth index (DMFT) ≥14 teeth] in most industrialized countries, with a lower prevalence in developing countries (Petersen et al., 2005). Regarding children, dental caries affects 60-90% of school-aged children in industrialized countries with the highest severity in 12-year old children in the Americas (DMFT= 3.0) followed by Europe (DMFT= 2.6) (Bratthall, 2005; Petersen et al., 2005).

Gingivitis is universally present in adults and approximately 53% of dentate adults in the United States have a life-experience of periodontal attachment loss of >3 mm
(Albandar, 2002). In children, gingivitis is also highly prevalent with a tendency to increase from infancy to puberty, but periodontitis is rare and the prevalence of aggressive periodontitis cases varies between 0.1% and 2.6% (Jenkins & Papapanou, 2001). Incipient periodontitis in children, however, may lead to periodontitis in adulthood (Bimstein, 1991; Kamma et al., 2000; Tanner et al., 2002).

**Bacterial Etiology of Dental Diseases - Hypotheses**

Different hypotheses have been proposed for the bacterial etiology of dental diseases. These theories have developed through the improved understanding of dental biofilm composition and organization. They also reflect the development of appropriate laboratory methods to identify these communities. Investigators from 1880 to 1930 suggested that possible etiological factors of periodontitis were amebae, spirochetes, fusiforms, and streptococci (Socransky & Haffajee, 1994). The initial hypothesis (1950) established for the microbial etiology of dental disease was the “non-specific plaque hypothesis”, in which dental diseases were caused by a concerted effect of the total oral plaque microflora (Theilade, 1986). Subsequently, the “specific plaque hypothesis” was proposed to suggest that only a few species from the resident microbiota were responsible for dental diseases, which although present in dental health were detected less frequently and in lower levels than in disease conditions (Loesche, 1976; Socransky, 1977). Finally, the “ecological plaque hypothesis”, a modification of the specific plaque hypothesis, proposed that change in the local ecosystem could promote growth of certain dental plaque microorganisms and lead to their increased numbers and dental disease development in disease susceptible individuals (Marsh, 2003).

**Dental Caries – Caries Development and Risk Factors**

In 1890, the “father” of oral microbiology, W. D. Miller suggested that oral bacteria are able to metabolize dietary carbohydrates and convert them into acid (Levine, 1977). The invariable role of bacterial infection combined with high sucrose diet in dental caries was demonstrated in experiments on hamsters (Gibbons & Keyes, 1969). A byproduct of acidogenic bacterial metabolism, lactic acid, lowers the pH locally and leads to enamel dissolution (Higuchi et al., 1969). Clinically, the initial loss of tooth mineral is reversible by remineralization, for instance by topical fluoride
treatment, decrease of sugar consumption, reduction of cariogenic bacteria or of bacterial ability to produce acid. However, if acidic challenge continues disease may progress into the adjacent dentin and the pulpal tissue causing endodontic infections (Fgdor & Sundqvist, 2007) and possibly leading to necrotic dental pulps.

Apart from bacteria and fermentable carbohydrates, additional risk factors for dental caries include behavioral factors such as poor oral hygiene (Axelsson & Lindhe, 1981a; Axelsson & Lindhe, 1981b; Axelsson et al., 2004), reduced salivary secretion and buffering capacity (Almstahl & Wikstrom, 1999), and genetic predisposition (Jonasson et al., 2007; Stenudd et al., 2001). Previous caries experience and presence of visible plaque have been identified as reliable predictors of increased caries risk in children (Alaluusua & Malmivirta, 1994). Caries risk assessment packages, such as Cariogram and Caries-risk Assessment Tool (CAT), have been designed for use in clinical environments to identify patients needing dental caries prophylaxis (American Academy of Pediatric Dentistry, 2005; Petersson & Bratthall, 2000).

**Dental Caries in Children**

Childhood dental caries is a major public health issue and is the most common chronic disease in children (Edelstein & Douglass, 1995). Despite the availability of prophylactic measures and treatment, approximately a third of pre-school children are affected by dental caries in the United States and Sweden (Beltran-Aguilar et al., 2005; Stecksen-Blicks et al., 2008; Wendt et al., 1992).

Caries in primary teeth of young children has been termed “baby bottle tooth decay”, “nursing caries” and “night bottle mouth” (Harris et al., 2004). The need for development and adoption of standardized definitions and criteria for the diagnosis of dental caries in primary teeth has been apparent for years. In 1997, new definitions for childhood caries were proposed at a National Institutes of Health (NIH) Workshop for diagnosis and research purposes (Drury et al., 1999). Later these guidelines were also adopted by the American Academy of Pediatric Dentistry (AAPD) (Reference Manual 2004-2005, www.aapd.org) and have been widely used in clinical studies in recent years. These definitions for Early Childhood Caries (ECC) and Severe Early Childhood Caries (S-ECC) are explained below (Table 1).
Table 1. National Institutes of Health workshop definitions of Early Childhood Caries (ECC) and Severe Early Childhood Caries (S-ECC) (Drury et al. 1999)

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Early Childhood Caries (ECC)</th>
<th>Severe Early Childhood Caries (S-ECC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-35</td>
<td>1 or more dmf surfaces †</td>
<td>1 or more smooth dmf surfaces †</td>
</tr>
<tr>
<td>36-47</td>
<td>1 or more dmf surfaces †</td>
<td>1 or more cavitated, filled or missing (due to caries) smooth surfaces in primary maxillary anterior teeth or dmfs score ≥ 4</td>
</tr>
<tr>
<td>48-59</td>
<td>1 or more dmf surfaces †</td>
<td>1 or more cavitated, filled or missing (due to caries) smooth surfaces in primary maxillary anterior teeth or dmfs score ≥ 5</td>
</tr>
<tr>
<td>60-71</td>
<td>1 or more dmf surfaces †</td>
<td>1 or more cavitated, filled or missing (due to caries) smooth surfaces in primary maxillary anterior teeth or dmfs score ≥ 6</td>
</tr>
</tbody>
</table>

† Any carious lesion, non-cavitated decayed (d1) or cavitated decayed (d2), missing tooth due to caries (m), or filled surface (f). Includes primary teeth only.

Microbiota of Dental Caries in Children

Mutans Streptococci

There is extensive evidence associating *Streptococcus mutans* and *Streptococcus sobrinus* (mutans streptococci) with dental caries. Specifically in children with ECC, the mutans streptococci can dominate the cultivable plaque microbiota associated with the lesions or the carious material itself (van Houte et al., 1982).

Mutans streptococci use sucrose to produce extracellular glucan, a water insoluble polysaccharide, which enables the bacteria to attach to the tooth surface and also protects them from external factors such as mechanical disruption, salivary clearance, and antimicrobial substances (Tinanoff et al., 2002). Equally important is the ability of mutans streptococci to both produce acid (acidogenic) and survive in an acidic environment (aciduric), properties that enable them to exhibit high pathogenicity (Svensater et al., 1997). Finally, mutans streptococci can survive when external carbohydrates are not present due to their ability to store intracellular polysaccharide (Takahashi et al., 1991).

Pre-school children with high levels of salivary mutans streptococci have greater caries prevalence and greater risk for new lesions compared with children with lower
mutans streptococci levels (O'Sullivan & Thibodeau, 1996). Studies have also indicated that the earlier mutans streptococci colonize the oral cavity of children, the higher their caries experience is (Alaluusua & Renkonen, 1983). On the other hand, several studies have failed to detect *S. mutans* in a significant portion of the children who develop caries (Aas *et al.*, 2008; Dasanayake & Caufield, 2002; Graves *et al.*, 1991), suggesting that additional species may play a significant role in dental caries development in children.

**Lactobacillus and Other Species**

In children with dental caries *Lactobacillus fermentum, Lactobacillus rhamnosus,* and *Lactobacillus casei* are prevalent in nursing caries lesions by culture methods (Marchant *et al.*, 2001). *Lactobacillus* species’ numbers in saliva seem to reflect the amount of simple carbohydrate consumption by the host (Tanzer *et al.*, 2001). Non-mutans streptococci, *Actinomyces,* and *Veillonella* species are found in high amounts in supragingival plaque and may comprise over one half of the cultivable flora. Gram-positive species of other genera with acidogenic potential found less frequently in plaque include *Bifidobacterium, Clostridium, Eubacterium, Propionibacterium,* and *Rothia* species (van Houte, 1994).

The concept that enamel and dentin demineralization can be due to other microorganisms apart from mutans streptococci and lactobacilli, has been the focus of recent research. Svensäter and co-workers showed in adult participants that 50% of the total cultured plaque microbiota from caries and health was able to grow at pH 5.5. At pH 5.0, *Streptococcus* species were the dominant group, but mutans streptococci accounted for less than half of the viable streptococcal count (Svensater *et al.*, 2003).

Molecular techniques have aided in precise bacterial identification and enumeration in dental caries (Munson *et al.*, 2004). By 16S rDNA cloning and sequencing, analysis of bacterial species in ECC identified 10 novel uncultivated phylotypes (Becker *et al.*, 2002). By oligonucleotide DNA probes, bacterial species associated with caries in order of decreasing cell numbers were: *Actinomyces gerencseriae,* *Bifidobacterium* species, *S. mutans,* *Veillonella* species, *Streptococcus salivarius,* *Streptococcus constellatus,* *Streptococcus parasanguinis,* and *L. fermentum* (Becker
et al., 2002). Another study, using both culture and molecular methods, indicated that the taxa most often isolated from ECC lesions (pH 5.2) were *Streptococcus oralis, S. mutans, Actinomyces israelii*, and *Actinomyces naeslundii* (Marchant et al., 2001). Other bacteria identified by molecular approaches and that are associated with childhood caries include non-mutans *Streptococcus, Lactobacillus, Actinomyces, Bifidobacterium* and *Veillonella* species (Aas et al., 2008; Corby et al., 2005). Further studies are needed to complete the knowledge regarding the array of bacterial species and phylotypes associated with ECC and S-ECC.

**Periodontal Diseases and Risk Factors**

Periodontal diseases, gingivitis and periodontitis, are inflammatory processes in tooth supporting tissues. They are frequently caused by the host defense factors induced by bacterial infection (Darveau et al., 1997; Kornman et al., 1997). Gingivitis involves an inflammatory response to dental plaque biofilms growing adjacent to the gingival margin. Plaque-induced gingivitis is reversible, if optimal oral hygiene is achieved, and the gingival tissue can return to its homeostatic state without permanent damage (Loe et al., 1965). Periodontal connective tissue attachment is lost in periodontitis, as opposed to gingivitis. Host defense against bacterial insult leads to apical migration of the junctional epithelium at the base of the gingival crevice. This process coupled with gingival edema, due to inflammatory response, leads to deepened periodontal pockets. In addition, in periodontitis the surrounding tissue (bone and connective tissue) of the teeth is destroyed in an attempt to limit the inflammatory process (Page & Kornman, 1997). The presence and extent of periodontal fiber loss can be determined by probing, which is the routinely used clinical method to estimate the extent of tissue destruction in periodontitis. Marginal alveolar bone loss can be diagnosed radiographically.

The classical Koch’s postulates for defining causative pathogens, have been amended in the case of periodontitis to include an evaluation of the evidence concerning association, elimination, host response, virulence factors, animal studies, and risk assessment of each potential pathogen (Fredericks & Relman, 1996; Haffajee & Socransky, 1994). Other factors associated with periodontitis, apart from bacterial colonization, include: compromised immunity (Cainciola et al., 1977), cigarette smoking (Haber et al., 1993), and diabetes mellitus (Kinane & Bouchard, 2008).
Microbiota of Periodontal Health and Disease

Periodontally healthy sites exhibit a low cultivable bacterial load by individual sulcus \((10^2 \text{ to } 10^3 \text{ microorganisms})\) with a predominantly Gram-positive microbiota, including *Streptococcus* and *Actinomyces* species (Table 2) (Darveau et al., 1997; Syed & Loesche, 1978; Tanner et al., 1996). Gingivitis is characterized by an increased microbial load \((10^4 \text{ to } 10^5 \text{ microorganisms by periodontal sulcus})\) as well as an increased prevalence of Gram-negative bacteria (15-50%) (Table 2) (Lai et al., 1987; Moore et al., 1987; Slots, 1979).

The most extensively studied periodontal pathogen which is associated with aggressive forms of periodontal destruction is *Aggregatibacter* (previously *Actinobacillus* *actinomycetemcomitans*). This small non-motile, capnophilic Gram-negative coccobacillus has been identified as the main causative agent of aggressive periodontitis in young individuals, but has also been found in adults (Asikainen, 1986; Asikainen et al., 1986; Slots et al., 1980; Tanner et al., 1979). *A. actinomycetemcomitans* has been grouped into six serotypes (Kaplan et al., 2001; Saarela et al., 1992; Zambon et al., 1983) and it has been suggested that certain serotypes are associated more frequently with periodontitis than periodontal health. Exemplifying this relationship, serotype c has been detected more frequently from periodontally healthy individuals and serotypes a and b more frequently in periodontitis (Asikainen et al., 1991; Dogan et al., 1999; Yang et al., 2005; Zambon et al., 1983). Although differences are reported in *A. actinomycetemcomitans* serotype distribution when taking geographic location and/or ethnicity into account (Dahlen et al., 2002; Dogan et al., 2003), still 3-8% of strains have remained non-serotypeable (Paju et al., 2000).

Gram-negative asaccharolytic obligate anaerobes *P. gingivalis*, *T. forsythia*, and *T. denticola* (Socransky et al., 1988) have been also extensively associated with periodontitis. *P. gingivalis* has been detected in association with periodontal lesions (Dahlen et al., 1992; Loesche et al., 1985; Moore et al., 1983) and possesses an arsenal of virulence factors that can potently activate the host response (Holt & Ebersole, 2005; Kuramitsu, 2003). *T. forsythia* was first described at the Forsyth Institute and soon became a recognized periodontitis pathogen due to its frequent detection from sites with destructive periodontitis and its strong association with
increasing pocket depth (Gmur et al., 1989; Lai et al., 1987; Tanner et al., 1979; Tanner & Izard, 2006). Finally, spirochetes, such as *T. denticola*, are also frequently found in periodontally diseased sites subgingivally and decrease after successful treatment (Simonson et al., 1988; Simonson et al., 1992; Socransky et al., 1969).

Table 2. Dominant bacterial species detected in periodontal health, gingivitis, and periodontitis. Species are ordered according to likelihood of detection by culture in each category starting from the most likely and proceeding to the least likely detected. (Adapted from Darveau et al., 1997)

<table>
<thead>
<tr>
<th>Periodontal Health</th>
<th>Gingivitis</th>
<th>Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus oralis</em></td>
<td><em>Streptococcus oralis</em></td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td><em>Streptococcus sanguinis</em></td>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
</tr>
<tr>
<td><em>Streptococcus mitis</em></td>
<td><em>Streptococcus mitis</em></td>
<td><em>Tannerella forsythia</em></td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td><em>Streptococcus intermedia</em></td>
<td><em>Spirochetes</em></td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td><em>Capnocytophaga ochracea</em></td>
<td><em>Treponema denticola</em></td>
</tr>
<tr>
<td><em>Streptococcus anginosus</em></td>
<td><em>Capnocytophaga gingivalis</em></td>
<td><em>Prevotella intermedia</em></td>
</tr>
<tr>
<td><em>Streptococcus intermedius</em></td>
<td><em>Campylobacter gracilis</em></td>
<td><em>Prevotella nigrescens</em></td>
</tr>
<tr>
<td><em>Gemella morbillorum</em></td>
<td><em>Prevotella loescheii</em></td>
<td><em>Campylobacter rectus</em></td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Eubacterium nodatum</em></td>
<td><em>Fusobacterium nucleatum subspecies vincentii</em></td>
</tr>
<tr>
<td><em>Actinomyces gerencseriae</em></td>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Fusobacterium nucleatum subspecies nucleatum</em></td>
</tr>
<tr>
<td><em>Actinomyces odontolyticus</em></td>
<td><em>Actinomyces israelii</em></td>
<td><em>Selenomonas noxia</em></td>
</tr>
<tr>
<td><em>Parvimonas micra</em></td>
<td><em>Campylobacter concisus</em></td>
<td><em>Selenomonas fluggeii</em></td>
</tr>
<tr>
<td><em>Eubacterium nodatum</em></td>
<td><em>Actinomyces odontolyticus</em></td>
<td><em>Enteric species</em></td>
</tr>
<tr>
<td><em>Capnocytophaga ochracea</em></td>
<td><em>Fusobacterium nucleatum subspecies nucleatum</em></td>
<td><em>Filifactor alocis</em></td>
</tr>
<tr>
<td><em>Capnocytophaga gingivalis</em></td>
<td><em>Eubacterium brachy</em></td>
<td><em>Lactobacillus uli</em></td>
</tr>
<tr>
<td><em>Campylobacter gracilis</em></td>
<td><em>Eikenella corroden</em></td>
<td><em>Veillonella parvula</em></td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum subspecies polymorphum</em></td>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
<td></td>
</tr>
</tbody>
</table>

Other species that have been associated with periodontitis include *Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia*, *Prevotella nigrescens*, *Campylobacter rectus*, *Eikenella corroden*, *Selenomonas* and *Eubacteria* species (Haffajee & Socransky, 1994; Moore & Moore, 1994) and more recently *Filifactor alocis* (Table 2) (Dahlen & Leonhardt, 2006; Hutter et al., 2003; Kumar et al., 2006). Recently, molecular microbiological studies have expanded the series of bacteria recognized in association with periodontal diseases to include uncultivated and less often identified phylotypes (Kumar et al., 2006; Kuramitsu, 2003; Paster et al., 2001).
Molecular Microbiological Identification of Oral Bacteria

Bacterial culture, particularly anaerobic culture, has been critical in the appreciation of the diversity of the subgingival microbiota. Use of non-selective and selective media and various atmospheric conditions, has aided in growth of multiple bacterial species from the oral cavity. Not all bacteria, however, present in oral samples have been cultured to date. The fact that certain microorganisms cannot be cultured and that culture has recognized limitations including sample transportation, time constraints, labor intensity, and high expense, has led to increasing use of molecular detection and identification methods. DNA based methods are currently being extensively used and renewed possibilities to study bacterial etiology of dental diseases have risen. DNA methods, such as PCR, DNA-DNA hybridization, 16S rDNA cloning and sequencing, and microarray analysis, allow simultaneous identification of numerous previously known bacteria and/or novel phyla and phylotypes (Table 3). These approaches providing the possibility of identification of novel pathogens associated with dental diseases.

Table 3. Molecular techniques and culture as methods of bacterial identification in oral samples. A comparison of strengths, limitations and application areas.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Limitations</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Cultured representatives</td>
<td>Time, cost, limited detection</td>
<td>Characterization, antibiotic resistance</td>
</tr>
<tr>
<td>PCR</td>
<td>Rapid, sensitive</td>
<td>Contamination, non-viable detection</td>
<td>Species present in low levels</td>
</tr>
<tr>
<td>Whole genomic DNA probes</td>
<td>Sensitive, quantitative</td>
<td>Specificity, cultured species</td>
<td>Multiple species</td>
</tr>
<tr>
<td>Oligonucleotide DNA probes</td>
<td>Specific, uncultivated</td>
<td>Sensitivity, primer bias</td>
<td>Multiple species, phylotypes</td>
</tr>
<tr>
<td>DNA microarray</td>
<td>Multiple species</td>
<td>Variance, multiple comparisons</td>
<td>Genetics, bacterial profiles</td>
</tr>
<tr>
<td>16S rDNA cloning and sequencing</td>
<td>Uncultivated</td>
<td>Time, cost, few samples</td>
<td>Phylogeny, diversity</td>
</tr>
</tbody>
</table>

In the human oral cavity, 700 bacterial species and phylotypes have been recently identified by molecular methods (Aas et al., 2005; Aas et al., 2008; Becker et al., 2002; Chhour et al., 2005; Corby et al., 2005; Kazor et al., 2003; Kroes et al., 1999; Paster et al., 2001; Preza et al., 2008). These molecular techniques open intriguing possibilities for identification of novel bacterial phylotypes (Table 4).
Table 4. Identification of novel bacterial phylotypes from dental caries or periodontitis in recent studies using 16S rDNA cloning and sequencing.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Subjects (Samples) (N)</th>
<th>Total Species/Phylotypes (N)</th>
<th>Novel Phylotypes (N)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caries (ECC)†</td>
<td>2 (5)</td>
<td>68</td>
<td>10</td>
<td>Becker et al. 2002</td>
</tr>
<tr>
<td>Caries</td>
<td>5 (5)</td>
<td>79</td>
<td>31</td>
<td>Munson et al. 2004</td>
</tr>
<tr>
<td>Caries</td>
<td>10 (10)</td>
<td>75</td>
<td>2</td>
<td>Chhour et al. 2005</td>
</tr>
<tr>
<td>Caries (S-ECC)†</td>
<td>7 (22)</td>
<td>197</td>
<td>12</td>
<td>Aas et al. 2008</td>
</tr>
<tr>
<td>Caries†</td>
<td>21 (43)</td>
<td>245</td>
<td>6</td>
<td>Preza et al. 2008</td>
</tr>
<tr>
<td>Periodontitis†</td>
<td>31 (31)</td>
<td>347</td>
<td>215</td>
<td>Paster et al. 2001</td>
</tr>
<tr>
<td>Periodontitis†</td>
<td>30 (45)</td>
<td>274</td>
<td>6</td>
<td>Kumar et al. 2005</td>
</tr>
</tbody>
</table>

† Studies include control group

Moreover, molecular methods also allow determining intra-species differences with higher reproducibility than phenotypic methods that rely on gene expression. In particular PCR-based methods, such as arbitrarily primed PCR (AP-PCR) and denaturing gradient gel electrophoresis (DGGE), have been widely used. Several studies have investigated the intra-species diversity of bacterial species associated with dental caries, such as *S. mutans*, *S. sobrinus* (Mattos-Graner et al., 2001; Saarela et al., 1996), and *Actinomyces* species (Ruby et al., 2003) and with periodontitis, such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, and *C. rectus* (Asikainen et al., 1995; Asikainen et al., 1996; Matto et al., 1996). Additionally, by PCR amplification of serotype-specific DNA sequences it is currently possible to group *A. actinomycetemcomitans* strains into serotype categories (Kaplan et al., 2001; Suzuki et al., 2001). These molecular techniques allow for more comparable studies between laboratories on the associations of different bacterial serotypes with dental diseases, due to the commercial availability of the reagents.

It has been estimated that half of the oral bacteria have not yet been cultured (Paster et al., 2001). Although 50% successful cultivation of bacteria in the oral cavity by far exceeds the 1% cultured on Earth (Hugenholtz et al., 1998), cultivation-based approaches have provided an incomplete picture of the microbial diversity of dental diseases and oral health. The conclusions drawn about bacteria associated with dental diseases have been dependent on the available technology. Molecular approaches provide the possibility of expanding the knowledge of the diverse array of microorganisms present in the oral cavity. In order to be able to identify candidate pathogens and beneficial species a comprehensive approach of the oral microbiota involved in oral health and disease is needed.
Hypothesis and Aims

The objective of this doctoral thesis was to investigate the role of the oral microbiota in dental caries and periodontitis using comprehensive molecular, clinical, and statistical methodology. The central hypothesis was that currently poorly characterized, fastidious, and uncultivated bacteria are involved in dental diseases.

Specific Aims

This doctoral thesis aimed by use of molecular methods:

1. To identify cultured caries- and periodontitis-associated species and determine their possible association with childhood caries.

2. To identify uncultivated species and novel phylotypes, determine their possible association with childhood caries, and examine the bacterial diversity of childhood caries.

3. To identify fastidious species and uncultivated phylotypes and determine their possible association with early periodontitis and periodontitis progression.

4. To investigate the reason for non-serotypeability of a subset of *A. actinomycetemcomitans* strains and their association with periodontitis.
Materials and Methods

Study Populations and Clinical Measurements

All study designs (Table 5), protocols, and informed consents were examined and approved by the Institutional Review Boards and/or the local ethical committees of the institutions involved.

Table 5. Summary of study design, origin of study populations, number and age of participants, dental disease category, and laboratory methods used for bacterial identification. The studies are presented in the order attached and discussed in the thesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Population (N of subjects)</th>
<th>Age (yrs)</th>
<th>Dental Disease</th>
<th>Laboratory Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cross-sectional</td>
<td>Boston, MA (N=195)</td>
<td>1-6</td>
<td>Caries (ECC)</td>
<td>Whole genomic probes, PCR</td>
</tr>
<tr>
<td>II</td>
<td>Cross-sectional</td>
<td>Boston, MA (N=80)</td>
<td>2-6</td>
<td>Caries (S-ECC)</td>
<td>16S rDNA cloning and sequencing, PCR</td>
</tr>
<tr>
<td>III</td>
<td>Cross-sectional</td>
<td>Boston, MA (N=141)</td>
<td>20-40</td>
<td>Early periodontitis</td>
<td>Oligonucleotide probes, multiplex PCR</td>
</tr>
<tr>
<td>IV</td>
<td>Longitudinal</td>
<td>Boston, MA (N=117)</td>
<td>20-40</td>
<td>Progressing periodontitis</td>
<td>Oligonucleotide probes, Whole genomic probes, multiplex PCR</td>
</tr>
<tr>
<td>V</td>
<td>Cross-sectional</td>
<td>Finland (N=152), Turkey (N=37)</td>
<td>13-74</td>
<td>Aggressive and chronic periodontitis</td>
<td>Immunodiffusion, SDS-PAGE, Western blot, PCR, AP-PCR</td>
</tr>
</tbody>
</table>

† Nested in a cohort

Childhood Caries (Study I and II)

Children with (N=35) and without (N=160) ECC were recruited from pediatric clinics, as part of a larger cohort study. Children with (N=40) and without (N=40) S-ECC were recruited from dental clinics in a case-control study (Study II). Inclusion criteria for both studies were that the child was up to 6 years old, medically healthy, had not used antibiotics for the past 3 months, and that the parent or guardian was willing to consent to the child’s clinical examination and microbial sampling. Children were examined by trained dental hygienists or dentists. Presence and status of teeth (Drury et al., 1999), plaque and gingival status were recorded. Bacterial samples were collected from anterior, posterior teeth, and the dorsum of the tongue. Children’s socio-demographic characteristics (Study I and II), diet, and oral health practices (Study I) were collected.
Early Periodontitis (Study III and IV)

Periodontally healthy subjects (N=28) and patients with early periodontitis (N=113) were recruited in this multicenter study (Study III). Recruitment methods and inclusion criteria have been previously described in detail (Tanner et al., 2005). Briefly, inclusion in the study was restricted to 20-40 year old subjects, who were medically healthy, and without recent antibiotic use or previous periodontal therapy. Clinical entry criteria included presence of at least 24 teeth, mean periodontal attachment loss of <2 mm, and no generalized gingival recession. Information concerning medical and dental histories, socio-demographic data, and tobacco use was collected. Clinically, duplicate measurements of probing depth and periodontal attachment level and single measurements of plaque index, gingival index, and bleeding on probing were obtained from six sites on all teeth (excluding third molars). Clinicians were trained and calibrated and exhibited intra-class correlations of 0.87 for pocket depth and 0.85 for clinical attachment level (Tanner et al., 2005). Monetary incentive was given to participants after completion of each clinical measurement visit.

A subset of participants (N=117) was followed longitudinally every 6 months for 18 months (Study IV). Subjects exhibiting ≥ 1 site with > 1.5 mm inter-proximal clinical attachment loss over 18 months of monitoring were labeled as “active”. “Inactive” subjects exhibited no sites with > 1.5 mm inter-proximal clinical attachment loss during the observation period.

Aggressive and Chronic Periodontitis (Study V)

Participants from Finland (N=152) and Turkey (N=37) were recruited to obtain 311 A. actinomycetemcomitans strains. Inclusion criteria were that patients were medically healthy, had no recent use of antibiotics, and were willing to partake in the study. The periodontal status of the subjects was determined clinically and radiographically.
Microbial Analysis

DNA-DNA Hybridization

Whole Genomic DNA Probe Assay

Plaque samples were analyzed in Studies I and IV by whole genomic DNA probes as previously described (Socransky et al., 1994). Briefly, samples were denaturated, neutralized, and fixed onto a membrane by ultraviolet (UV) irradiation. The two last lanes on each membrane were used as a quantitative reference containing all probe species mixed as a DNA standard for $10^5$ and $10^6$ bacterial cell equivalents. The membrane containing the samples and standards was hybridized with digoxigenin-labeled probes directed to the species of interest. Finally, anti-digoxigenin antibodies, covalently bound to alkaline phosphatase, were used to visualize the final product with standard chemiluminescence. Signals were detected using a Storm Fluorimager system (Molecular Dynamics, Sunnyvale, CA) and subsequently converted to absolute bacterial counts by comparison with the standards on the membrane. The sensitivity of the assay was set to $10^4$ bacterial cells by adjusting the concentrations of the probes. The specificity of the assay was $<10\%$ cross-reactivity for 97.4% of probes, $<5\%$ cross-reactivity for 93.5% of probes, and $<1\%$ cross-reactivity for 82.1% of the probes (Socransky et al., 2004).

In Study I, samples were analyzed using whole genomic DNA probes to 74 species (Study I, Table 2), including 44 acidogenic and aciduric bacterial species (Becker et al., 2002; Marchant et al., 2001; Munson et al., 2004; Svensater et al., 2003) and 30 mainly Gram-negative bacterial species selected according to previously reported associations with dental health or disease.

For the initial periodontitis population (Study IV) a set of 38 whole genomic DNA probes was selected to include species identified from previous culture studies of initial periodontitis (Tanner et al., 1998).

Oligonucleotide DNA Probe Assay

Samples were analyzed in Studies III and IV by 78 16S rDNA probes selected to include cultured species and uncultivated phylotypes previously associated with periodontitis (Kazor et al., 2003; Paster et al., 2001) in an assay as previously described (Paster et al., 1998). Briefly, the oligonucleotide DNA probes (17-22 bases)
were cross-linked to a nylon membrane using UV irradiation. Two universal standard probes were used for each membrane. DNA from samples was amplified with two universal primers, one of which was labeled with digoxigenin. The digoxigenin-labeled amplicon was subsequently hybridized to the capture probes and anti-digoxigenin antibodies, covalently bound to alkaline phosphatase, were used to visualize the final product by chemiluminescence. Signals were detected using a Storm Fluorimager system and reactions were scored as species detected or not detected.

**Polymerase Chain Reaction**

**S. mutans and A. actinomycetemcomitans PCR**

PCR for detection of *S. mutans* (Study I and II) and of *A. actinomycetemcomitans* (Study II) from samples was used to detect these specific dental pathogens from clinical samples. In Study I, samples were treated by Proteinase K (Dewhirst *et al.*, 2000) to inactivate proteases and the product was used as a template for the PCR. In Study II, genomic DNA was extracted and purified from samples as previously described (Li *et al.*, 2007). *A. actinomycetemcomitans* PCR (Study II) was performed in a nested format, by running a universal PCR first and then using the template for the PCR as previously described (Ashimoto *et al.*, 1996). To ensure correct amplification products, all *A. actinomycetemcomitans* positive reactions were sequenced.

**A. actinomycetemcomitans Genotyping PCR**

Arbitrarily primed PCR was performed (Study V) using the random oligonucleotide sequence OPA-13 (5’-CAG CAC CCC AC-3’) to separate *A. actinomycetemcomitans* strains into distinct genotypes as previously described (Asikainen *et al.*, 1995; Dogan *et al.*, 1999; Paju *et al.*, 2000).

**A. actinomycetemcomitans Serotype-specific PCR**

The gene sequences specific for *A. actinomycetemcomitans* serotypes a through f were assayed (Study V) as previously described (Dogan *et al.*, 2003; Kaplan *et al.*, 2001; Suzuki *et al.*, 2001). Moreover, the entire serotype-specific gene clusters were analyzed for serotype a, c, and f.
**P. gingivalis and T. forsythia Multiplex PCR**

To improve detection of the two major periodontitis-associated pathogens *P. gingivalis* and *T. forsythia* (Study III, IV) a multiplex PCR was performed based on the PCR assay by Tran and Rudney (Tran & Rudney, 1999). The detection limit was for *P. gingivalis* $10^2$ and for *T. forsythia* $10^3$.

**Cloning and Sequencing**

Genomic DNA was extracted from the samples using a previously described protocol (Li et al., 2007). PCR amplification of the 16S rRNA gene, and cloning and sequencing were performed (Study II) as previously described (Paster et al., 2001) using universal PCR primers under standard conditions (Dewhirst et al., 1999). Cloning of the PCR-amplified DNA was performed with a commercial kit (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Transformation was performed in competent One Shot TOP 10 *E. coli* cells (Invitrogen) overnight on Luria-Bertani agar plates supplemented with kanamycin. An X-galactose screen determined successful transformation.

Forty-eight to ninety-six transformed colonies were used as PCR templates with vector primers M13F and M13R (TOPO TA Cloning kit, Invitrogen, CA). Colonies with the correct insert size (1,600 bases) were cycle-sequenced (Big Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS, Perkin-Elmer, Waltham, MA) with 533 reverse primer (Paster et al., 2001). After a final clean-up, reactions were sequenced using an ABI 3100 DNA Sequencer.

Sequences were edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI), aligned, and checked individually for chimeric inserts. Sequences were compared using BLAST (Basic Local Alignment Search Tool) to a reference set of sequences in the Human Oral Microbiome Database, with a threshold for species differentiation >98% identity (Dewhirst et al., 2008). Neighbor-joining phylogenetic trees were created from full 16S rRNA Human Oral Microbiome Database reference sequences by DNASTAR MegAlign software using the CLUSTAL V alignment method.
Antigen Identification

SDS-PAGE and Western Immunoblotting

For *A. actinomycetemcomitans* serotype antigen detection, SDS-PAGE and Western immunoblotting were performed (Study V) as previously described (Karched et al., 2008). Whole cell preparations in phosphate buffer solution (PBS) were used and selected preparations were treated with Proteinase K. Both rabbit antisera against *A. actinomycetemcomitans* serotypes a-f and human sera were used. Immunoreactive bands were detected by chemiluminescence.

Immunodiffusion Assay

Serotype-specific rabbit antisera against whole cells of *A. actinomycetemcomitans* serotypes a through e were used as antibodies as reported previously (Saarela et al., 1999), with the addition of serotype f (Study V). Antigens were prepared from each *A. actinomycetemcomitans* strain by autoclaving whole cell suspensions and using the supernatant in the immunodiffusion assay.

Statistical Analysis

Univariate Analysis

**Study I**: Student’s t-test was used to compare differences in means, Chi-square test for differences in proportions between the ECC and the caries-free group, and McNemar’s test for species differences in paired samples. A p-value ≤0.05 was considered significant. Results were adjusted for multiple comparisons using the false discovery rate (α=0.1), which is a non-conservative approach to control for false positive results using the proportion of false positives rather than the chance of any false positives (Benjamini & Hochberg, 1995). Statistical analyses were performed using SPSS® software version 11.

**Study II**: Species and phylotypes were grouped into phyla and the number of distinct identification in each phylum by Human Oral Taxon number (HOT) was compared between S-ECC and caries-free children (Dewhirst et al., 2008). Taxa that could not be differentiated by 500 base 16S rRNA sequence comparison were grouped together for analysis. Student’s t-test (clinical and socio-demographic variables) and Kruskall-Wallis test (bacterial variables) were used to determine differences in means between S-ECC and caries-free children. The Chi-square test
was used to test differences in proportions of socio-demographic variables. A p-value ≤0.05 was considered significant. Results were also adjusted for multiple comparisons using the false discovery rate (α=0.05) (Benjamini & Hochberg, 1995). Statistical analyses were performed using SPSS® software.

**Study III:** McNemar's test was used to evaluate differences in species prevalence in tongue and subgingival samples. Similarities in species detection in tongue and subgingival samples were evaluated by Chi-square test for association. Comparison of species prevalence across clinical groups was evaluated by Mantel-Haenszel Chi-square for trend. Statistical analyses were performed using SPSS® and SAS® software.

**Study IV:** Student’s t-test was used to evaluate differences between active and inactive subjects with respect to age and baseline clinical variables. Analysis of covariance adjusting for baseline values was used for clinical variable comparison (mean values over 18 months) across the groups. Baseline clinical variables and subsequent change were evaluated by Spearman’s rank correlation. Clinical associations of active and inactive disease were compared with the Mantel-Haenszel Chi-square for trend. For microbial analysis, species were compared between active and inactive groups by Chi-square test for the oligonucleotide DNA probes and by Student’s t-test for the whole genomic DNA probes. The Chi-square test was also used to evaluate the PCR data. Statistical analyses were performed using SPSS® and SAS® software.

**Multifactorial analysis**

**Study I:** Regression analysis was used to evaluate the extent of caries in the children with age and plaque, and their interactions using SPSS® software. Multivariate analysis was performed using partial least squares (PLS) modeling (SIMCA P, Umetrics, Umeå, Sweden) to find positive and negative bacterial associations with caries. PLS is a linear model that detects correlations between independent and outcome variables and generates a Variable Importance in Projection (VIP) value which if >1 is influential and if ≥1.5 is highly influential.

**Study IV:** Logistic regression was used to evaluate clinical measurements at baseline, *P. gingivalis* and *T. forsythia* PCR detection and subsequent loss of attachment using SAS® software.
Results and Discussion

Oral Microbiota in Patients with Childhood Caries (Study I and II)

Bacterial samples from 275 children (75 with caries and 200 caries-free) were examined (Study I and II). By whole genomic DNA probes, 74 cultured species were identified including acidogenic, aciduric, and periodontitis-associated species (Study I, Table 2). By 16S rDNA cloning and sequencing, 3802 clones yielded identifications representing 138 bacterial taxa (77 cultured named species, 11 cultured unnamed species, and 50 uncultivated phylotypes) (Study II, Figure 1). An additional 107 clones (39 from caries and 68 from caries-free children) remained unidentified and may include novel phylotypes. Full 16S rRNA analysis of the unidentified clones will be needed to determine novelty of species.

Species detected (N=37) by whole genomic DNA probes (Study I) and 16S rDNA cloning and sequencing analysis (Study II) in children with and without dental caries are presented in Table 6. The table also indicates whether these species were detected more frequently in children with ECC and S-ECC than caries-free children and whether the detection differed significantly between the comparison groups (caries versus caries-free). The species detected by both assays represent half of the species assayed by the probe assay (37/74, 50%) and included both Gram-positive and Gram-negative species. Species assayed by the whole genomic DNA probes were pre-selected, whereas 16S rDNA cloning and sequencing species/phylotype detection was random. Significant associations for bacterial species detection in children with caries compared to caries-free children were observed in 13 different species by the two molecular methods in the two populations (Table 6). The only species exhibiting a significant invert association with the caries and caries-free group by the two methods was Streptococcus intermedius associated with ECC (p<0.05) (Study I, Figure 1), but not with S-ECC (p<0.05) (Study II, Figure 3a). By culture methods, although S. intermedius dominated children’s dental plaque from initial caries, it did not significantly differ from caries-free sites (Svensater et al., 2003).
Table 6. Species detected (N=37) by both whole genomic DNA probes (Study I) and 16S rDNA cloning and sequencing (Study II). The table indicates whether species detection was more frequent from children with dental caries [Early Childhood Caries (ECC, Study I) or Severe Early Childhood Caries (S-ECC, Study II)] compared to caries-free controls. Significant differences of species between caries and caries-free children are also indicated.

<table>
<thead>
<tr>
<th>Species Detected by Both Assays</th>
<th>Whole Genomic DNA Probes</th>
<th>16S rDNA Cloning and Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECC p&lt;0.05</td>
<td>S-ECC p&lt;0.05</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>+ c</td>
<td>* d</td>
</tr>
<tr>
<td>Streptococcus sobrinus</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus oralis</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus mitis/pneumonia</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus sanguinis</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus parasanguinis</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus cristatus</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus gordonii</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus intermedius</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Streptococcus anginosus</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Lactobacillus vaginalis</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Lactobacillus fermentum</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Lactobacillus gasseri</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Gemella morbillorum</td>
<td>+</td>
<td>ns</td>
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<tr>
<td>Actinomyces israelii</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Actinomyces naeslundii</td>
<td>+</td>
<td>ns</td>
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<td>Eubacterium saburreum</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Eubacterium brachy</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Parvimonas micra</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Prevotella melaninogenica</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Prevotella nigrescens</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Capnocytophaga gingivalis</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Capnocytophaga ochracea</td>
<td>+</td>
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</tr>
<tr>
<td>Capnocytophaga sputigena</td>
<td>+</td>
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<tr>
<td>Aggregatibacter actinomycetemcomitans</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Neisseria mucosa/ flava/sicca</td>
<td>+</td>
<td>ns</td>
</tr>
<tr>
<td>Eikenella corrodens</td>
<td>+</td>
<td>ns</td>
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<tr>
<td>Kingella oralis</td>
<td>+</td>
<td>ns</td>
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<tr>
<td>Campylobacter concisus</td>
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<td>ns</td>
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<td>Campylobacter showae</td>
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<tr>
<td>Campylobacter gracilis</td>
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<tr>
<td>Veillonella parvula/dispar</td>
<td>+</td>
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<tr>
<td>Selenomonas sputigena</td>
<td>+</td>
<td>ns</td>
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<tr>
<td>Selenomonas noxia</td>
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<td>ns</td>
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<tr>
<td>Selenomonas flueggei</td>
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a Chi-square test, b Kruskall-Wallis test, c + = Species detected more frequently from caries group (ECC Study I, S-ECC Study II), d * = p<0.05, e - = Species detected more frequently from caries-free group, f ns= not significant, g na = not available (detected in one group)
Mutans and Non-mutans Streptococci

Studies examining dental caries often focus on presence and association of mutans streptococci, which is a collective name for *S. mutans* and *S. sobrinus*. In the present studies, *S. mutans* was associated with dental caries in children both with ECC (Study I) and with S-ECC (Study II). *S. mutans* was detected from molar plaque samples by PCR in high percentages of children in both study populations: 69% in ECC (p<0.001, compared to caries-free children) and 73% in S-ECC (p<0.01, compared to caries-free children) (Figure 1). It has been previously reported by culture that 35% of 8-15 month old children harbored *S. mutans*, with 14% of the children by 10 months and 60% by 15 months of age (Karn et al., 1998). Moreover, *S. mutans* has been shown to exceed 30% of the cultured microbiota in ECC (Berkowitz et al., 1984; Marchant et al., 2001; van Houte et al., 1982). *S. mutans*, however, was not associated with S-ECC by 16S rDNA cloning and sequencing (Study II, Figure 3a) and was detected at a mean detection frequency of 1% by child. The infrequent detection of *S. mutans* by 16S rDNA cloning and sequencing analysis in subjects with dental caries has been previously reported in both childhood and adult caries, as well as root caries (Aas et al., 2008; Chhour et al., 2005; Preza et al., 2008). This could suggest that *S. mutans* represents a subset of the caries-associated microbiota (Loesche & Syed, 1973) and additional species could be responsible for caries development. However, methodological limitations cannot be excluded.

![Figure 1](image.png)

**Figure 1.** *S. mutans* detection by PCR in children with ECC (Study I) and S-ECC (Study II). Panel A: *S. mutans* PCR detection by sampling site. Significantly higher detection of *S. mutans* from children with ECC than caries-free children was observed from molar and incisor teeth (p<0.001), but not from the tongue. Panel B: *S. mutans* detection was associated with S-ECC in molar teeth (p<0.01).
**S. sobrinus** was only associated with ECC together with **S. mutans** (Study I, Table 2) and was found in only one child (S-ECC) by 16S rDNA cloning and sequencing (Study II). Detection of **S. sobrinus** together with **S. mutans** by PCR has been previously associated with higher caries prevalence than for **S. mutans** alone in 3-5 year old children (Okada et al., 2005; Seki et al., 2006). Moreover, a previous report using 16S rDNA cloning and sequencing as well as oligonucleotide DNA probe analysis has also shown that **S. sobrinus** is present in lower levels than **S. mutans** in children and not associated with childhood caries (Becker et al., 2002).

Concerning non-mutans streptococci, **S. mitis/pneumoniae** and **Streptococcus** sp. HOT-071 were detected more frequently from S-ECC children than caries-free children (Study II, Figure 3a). Although these species did not exhibit a significant association with childhood caries in Study II, they are of interest since they were previously associated with childhood caries by oligonucleotide DNA probes (Becker et al., 2002). By 16S rDNA cloning and sequencing **Streptococcus** sp. HOT-071 was detected from carious root surfaces of adults (Preza et al., 2008). Further studies are needed to determine the role of this unnamed species in dental caries.

Several non-mutans streptococcus species that were detected more frequently in caries-free children than children with S-ECC (Study II, Figure 3a) included **Streptococcus cristatus** (p<0.05), **Streptococcus sanguinis** (p<0.05), **S. intermedius** (p<0.05), and **Abiotrophia defectiva** (p<0.01) [**A. defectiva** previously classified as an atypical **Streptococcus** species (Kawamura et al., 1995)]. These findings are consistent with molecular studies of the microbiota in caries-free children of preschool age (Aas et al., 2008; Becker et al., 2002; Corby et al., 2005). **S. sanguinis**, **S. intermedius**, and **A. defectiva** have also been identified as part of the commensal oral bacterial flora by 16S rDNA cloning and sequencing from samples of dentally healthy adults (Aas et al., 2005).

**Lactobacillus Species**

**Lactobacillus** species exhibiting association with childhood caries were **Lactobacillus gasseri** (p<0.01), **L. fermentum** (p<0.05), and **Lactobacillus vaginalis** (p<0.05) (Study I, Figure 1). Significant associations with the two first species have been found by use of 16S rDNA cloning and sequencing and oligonucleotide DNA probe
analysis in childhood caries (Aas et al., 2008; Corby et al., 2005). Although L. vaginalis has been found in the saliva of caries-active women (Caufield et al., 2007), it is usually considered to be part of the normal vaginal flora (Thies et al., 2007). Based on culture methods, Lactobacillus species can be transiently detected in the infant oral cavity, but do not persist to colonize (Carlsson & Gothefors, 1975). In S-ECC, L. fermentum, L. gasseri, L. vaginalis, and Lactobacillus paracasei were detected in low frequencies by child (0.06-0.12%) and were detected more frequently in caries-free children (Study II). No conclusions can be, however, drawn about the role of these species in S-ECC due to the low detection frequency.

Noteworthy was the finding that both Lactobacillus acidophilus and Lactobacillus reuteri were found to be consistently influential by multifactorial analysis (VIP>1.0) in children with low decayed filled teeth index (dft), exhibiting an inverse relationship with dental caries (Study I). On restricting the analysis to children with visible plaque, L. acidophilus (VIP>1.5) became highly negatively influential on caries extent (dft) and L. reuteri, L. rhamnosus, and Lactobacillus plantarum exhibited negative influential associations with caries (VIP 1.0-1.4) (Study I, Figure 2). L. acidophilus and L. reuteri have been used for probiotic enteric therapy in infants (Lee et al., 2007; Savino et al., 2007). All four species have also been tested as oral anti-caries agents in children (Twetman & Stecksen-Blicks, 2008), and are consistently reported as beneficial species not associated with dental caries.

Other Species

A. israelii (p<0.05) and Actinomyces odontolyticus (p<0.05) were detected more frequently from children with caries than caries-free children by whole genomic DNA probes (Study I, Figure 1). Both species were highly influential with caries extent (higher dft scores) by multifactorial analysis (VIP>1.0) in all ages. When restricting the analysis to children with visible dental plaque, A. israelii remained highly influential with caries extent (VIP>1.0) and A. odontolyticus was borderline influential (VIP=0.9) (Study I, Figure 2). A previous study focusing on the predominant cultured microbiota of interproximal surfaces in children found that A. israelii was the most predominant of all Actinomyces species and that A. odontolyticus was the most prevalent species below the contact area of the teeth.
A. israelii has been found more frequently associated with dental plaque in children than with caries lesions by culture (Marchant et al., 2001). A. israelii has been previously detected in children younger than 18 months using the whole genomic DNA probe assay (Tanner et al., 2002). These findings have not been previously reported by oligonucleotide DNA probes (Aas et al., 2008; Corby et al., 2005; Tang et al., 2003). This possibly reflects a limitation of PCR based methods to amplify representatives of the Actinobacterium phylum, which contains Gram-positive species with high 16S rRNA G+C content. For detection of Actinomyces species, the whole genomic DNA probe technique used in Study I was a more preferable approach than molecular methods utilizing amplification of the 16S rRNA gene (such as oligonucleotide DNA probes or 16S rDNA cloning and sequencing).

Veillonella parvula (p<0.01) (Study I, Figure 1), Veillonella atypica (p<0.05), and Veillonella sp. HOT-780 (p<0.01) (Study II, Figure 3a) were associated with dental caries in the childhood populations examined. Frequent association with Veillonella species and caries, especially childhood caries, have been shown both by culture (Marchant et al., 2001) and by molecular methods (Becker et al., 2002). Veillonella species have also been shown to dominate the oral microbiota of all stages of caries cavity formation in both primary and secondary teeth (Aas et al., 2008). Although Veillonella species are weak acid producers and probably not involved in the demineralization process of enamel or dentin, they may act as acid scavengers using lactate as a carbon source (Egland et al., 2004).

In terms of detection frequency and mean detection of bacteria in childhood caries, the most frequently detected species (>60% of the children) included Streptococcus and Actinomyces species, R. dentocariosa, F. alocis, and V. parvula (Study I, Table 2, Figure 1). Moreover, significantly higher mean levels were observed in children with ECC compared to caries-free children for A. naeslundii (p<0.001), A. odontolyticus (p<0.001), A. israelii (p<0.05), A. gerencseriae (p<0.05), V. parvula (p<0.001), and F. nucleatum subspecies polymorphum (p<0.05) (Study I, data not shown). A. odontolyticus (p<0.05), A. israelii (p<0.05), and V. parvula (p<0.01) were detected significantly more frequently in children with caries than in caries-free children (Study I, Figure 1). By 16S rDNA cloning and sequencing the majority of
clones detected by child included *Veillonella, Streptococcus*, and *Capnocytophaga* species and novel phylotypes that were detected as a lower percentage of the individual microbiota, possibly explaining the lack of association with childhood caries (Study II, Figure 3a).

**Periodontitis-associated Bacteria**

Study I included a DNA probe panel for detection of additional bacterial species associated with periodontal diseases (Study I, Table 2). Fastidious periodontal pathogens found more frequently in children with caries than in caries-free children included *A. actinomycetemcomitans* (p<0.05), *P. micra* (p<0.05), *P. gingivalis* (p<0.01), *Eubacterium brachy* (p<0.05), and *F. alocis* (p<0.05) (Study I, Figure 1). Presence of periodontal pathogens in young children has been previously reported by culture in intact teeth and by DNA probes in dental caries (Kamma *et al.*, 2000; Tanner *et al.*, 2002). *E. brachy* and *F. alocis* are species not frequently reported in children but have been associated with periodontitis in adults both by culture, DNA probes, and 16S rDNA cloning and sequencing (Dahlen & Leonhardt, 2006; Hill *et al.*, 1987; Kumar *et al.*, 2006).

*A. actinomycetemcomitans* by 16S rDNA cloning and sequencing was present in 15% of children by PCR (Study II, Figure 3b) and was not associated with S-ECC (Study II, Figure 3a). The detection frequency of the species was similar to that previously described in young children by culture and PCR in Europe and the United States (Alaluusua & Asikainen, 1988; Ashimoto *et al.*, 1996). Notably, in children and adolescents originating from Africa, the prevalence of the species (69%) and of particular clones of the species exhibiting higher pathogenic potential was higher (Haubek *et al.*, 1996; Haubek *et al.*, 2008). The use of PCR for both *A. actinomycetemcomitans* and *S. mutans* improved detection of these species present in low levels of the microbiota.

**Socio-demographic, Dietary, and Clinical Factors**

Clinical and background variables other than microbial composition associated with childhood caries included race (p<0.05), previous dental visit (p<0.05), cracker, chip, and cereal consumption (all p<0.05), and visible plaque (p<0.001) (Study I, Table 1). Black and Asian children exhibited a higher prevalence of dental caries.
than children of other races as has also been observed previously in the United States (Beltran-Aguilar et al., 2005; Shiboski et al., 2003). Snacking has been previously associated with ECC, and especially snacking on high starch foods, like chips and crackers that can be retained on the tooth surfaces (Kashket et al., 1996). Previous dental visit was associated with presence of dental caries, since families with children who experience dental pain are those who seek dental treatment. Finally, presence of visible dental plaque, which exhibited the highest association with childhood caries (Study I, Table 1), has been previously strongly associated with childhood caries (Mohebbi et al., 2006; Wennhall et al., 2002) and serves as a predictor for future caries development (Alaluusua & Malmivirta, 1994) in young children.

Age and presence of plaque (Study I) exhibited a linear relationship and a significant interaction effect on caries levels (dft) ($\beta$age*plaque=0.495, $p<0.05$) (Figure 2). Nonetheless, species associated with ECC and caries-free children in comparisons of all children and children with visible plaque were similar (Study I, Figure 2).

Figure 2. Extent of dental caries (dft: decayed filled teeth index) in relation to child age. The scatter plot of dft versus age in the presence of plaque illustrates that age and plaque exhibited a significant interaction effect on caries extent ($\beta$age*plaque=0.495, $p<0.05$).
Oral Microbiota of Early Adult Periodontitis (Study III and IV)

The oral microbiota associated with early adult periodontitis was examined in Studies III and IV. A total of 141 individuals participated in Study III comprised of 28 periodontally healthy subjects, 71 with Early Periodontitis 1 (subjects with mean attachment level ≤1.5 mm and at least one site ≥ 2 mm attachment loss), and 42 with Early Periodontitis 2 (subjects with mean attachment level >1.5 mm and mean attachment loss ≥ 2 mm) (Study III, Table 1). One hundred seventeen individuals were followed over 18 months and 19% (22/117) exhibited active periodontal breakdown by losing >1.5 mm clinical attachment in one or more interapproximal sites during the observation time (Study IV).

Use of oligonucleotide DNA probes (Study III, IV) allowed identification of phylotypes with no cultured representatives, such as TM7 and Obsidian Pool phylotypes (Hugenholtz et al., 1998), and fastidious species, such as treponemes and F. alocis (Study III, Figures 1, 2 and Study IV, Figure 4).

Microbiota of Early Periodontitis and Periodontally Healthy Subjects

The most frequently detected species (>60% of the subjects) included Streptococcus, Fusobacterium, and Granulicatella species (Study III, Figure 2). Interestingly, most of the uncultivated phylotypes identified were detected in >20% of subjects (Study III, Figure 2).

Species detected more frequently in early periodontitis than in periodontally healthy subjects included T. denticola, F. alocis, Porphyromonas endodontalis, Bacteroides sp. HOT-274 (oral clone AU126), F. nucleatum, and A. odontolyticus (p<0.01) by oligonucleotide DNA probes (Study III, Figure 2) and P. gingivalis (p<0.001) and T. forsythia (p=0.03) by PCR (Study III, Figure 3). In previous studies, T. denticola, F. alocis, and Bacteroides sp. HOT-274 (oral clone AU126) have been detected more frequently in periodontitis than in health (Paster et al., 2001). Moreover, by means of oligonucleotide DNA probes the previously reported species with the addition of P. endodontalis were associated with periodontitis (Kumar et al., 2003). F. alocis and P. endodontalis, both strict anaerobes have likely been underrepresented by culture of periodontitis samples, due to their fastidious growth requirements. It has been recently proposed to include
these two species in the panel of species used for periodontitis diagnostic purposes (Dahlen & Leonhardt, 2006).

The significant association of *P. gingivalis* with early periodontitis compared to oral health (p<0.001) (Study III, Figure 3) and with periodontal disease progression compared to stability (p<0.001) (Study IV, Figure 7) in these two studies was notable. In previous culture studies of initial periodontitis, our group had not found an association of *P. gingivalis* with periodontitis (Tanner et al., 1996). Few studies have examined early or incipient periodontitis in adults. The literature, thus, mainly supports identification of the species in more advanced and chronic periodontitis both by culture and molecular methods (Hamlet et al., 2001; Slots, 1986; Zambon et al., 1981).

*T. forsythia* detection by PCR was only associated with early periodontitis at baseline (p=0.03) (Study III, Figure 3) and not at follow-up analysis (p=0.07) (Study IV, Figure 7). Tanner and co-workers have previously detected *T. forsythia* in association with initial periodontitis by culture (Tanner et al., 1996). In a longitudinal prospective study of adults using the same PCR method as the current study, persistent detection of *T. forsythia* was predictive of progression of periodontal attachment loss (Tran et al., 2001).

*A. actinomycetemcomitans* was not significantly associated with initial periodontitis (Study III) or with periodontitis progression (Study IV), although it was detected at 24% from subjects exhibiting and at 21% from subjects not exhibiting attachment loss (Study IV, Figure 4). Detection frequency was similar at baseline (Study III) and corresponded to detection frequency by culture (27%) of 18-25 year old men with minimal attachment loss (Muller et al., 1996). Since the methods used did not allow for specific serotype identification of *A. actinomycetemcomitans*, the strains identified could also have been of serotypes previously associated with periodontal health (Asikainen et al., 1995; Zambon et al., 1983). Thus, it might be pivotal to identify specific clones or serotypes of bacteria associated with either periodontal health or disease.
**Subgingival and Tongue Surface Microbiota**

There were distinct differences by oligonucleotide DNA probes in species detection between bacterial samples from subgingival sites and the tongue surface (Study III, Figure 1). Species detected more frequently from the tongue surface were mainly Gram-positive *Streptococcus*, *Gemella* and *Granulicatella* species (Study III, Figure 1). Subgingival samples exhibited a higher prevalence of periodontitis-associated species than samples from tongue surface, including *A. actinomycetemcomitans*, *T. denticola*, *T. forsythia*, *F. alocis*, and *P. endodontalis* (all p<0.05), and uncultivated phylotypes, including TM7, *Treponema* and *Actinobaculum* clones (all p<0.05) (Study III, Figure 1). However, several species detected more frequently in periodontitis patients than healthy controls in previous studies, also exhibited higher detection frequency from tongue surfaces in the present study; for example *Eubacterium sulci* (Han et al., 1991), *Camplylobacter concisus* (Kamma & Nakou, 1997; Macuch & Tanner, 2000), *Megasphera* (Kumar et al., 2003; Kumar et al., 2006), and Obsidian Pool phylotypes (Kumar et al., 2003; Li et al., 2006).

Although *P. gingivalis* (by PCR) was detected equally frequently from the subgingival and the tongue surface samples, *T. forsythia* was detected more frequently subgingivally (p<0.001) (Study III, Figure 3). Similarly Dahlén and co-workers, identified *P. gingivalis* as a species that could significantly discriminate between sites with and without periodontitis even from the dorsum of the tongue (Dahlen et al., 1992). However, examining our overall results, most Gram-negative species were detected with a higher frequency from subgingival than tongue plaque samples (Study III, Figure 1) using oligonucleotide DNA probes. Similarly, in the study of childhood caries (Paper I) the detection frequency of all species assayed was lower in plaque samples taken from the dorsum of the tongue than the tooth surfaces utilizing the whole genomic DNA probes.

**Progressing Chronic Periodontitis**

Gram-positive species *Streptococcus infantis*, *S. sanguinis*, and *S. parasanguinis*, and the Gram-negative *F. nucleatum* (all p<0.03) were the species detected significantly at a higher frequency from early periodontitis subjects exhibiting attachment loss during follow-up than from “inactive” subjects (Study IV, Figure 4). These finding are consistent with the fact that subgingival plaque forms from apical
migration of the plaque growing on the tooth surface supragingivally (Theilade & Theilade, 1985). In incipient periodontitis it could be expected that the associated Gram-negative microbiota has not yet been established.

Detection of uncultivated phylotypes (Study IV, Figure 4) such as the Obsidian Pool, TM7, Actinobaculum, Megaspheira, Eubacterium, Porphyromonas, Bacteroidetes, and Treponema clones, although not statistically associated with periodontal destruction activity, exemplify how complex and diverse the subgingival microbiota is, which is not always apparent from culture studies. Higher detection frequency of periodontitis-associated species including P. micra and F. alocis by whole genomic DNA probes and T. forsythia and P. gingivalis by PCR (p=0.0073) was observed in subjects that exhibited attachment loss compared to those that did not (Study IV, Figures 5 and 7).

The fact that few bacterial species were associated with early periodontitis or disease progression (Study III, IV) could be due to low bacterial numbers in shallow pockets. In addition, during the follow-up period, supragingival plaque was removed before subgingival plaque sampling (Study III) and subjects were offered professional cleaning after each sampling. This could also have affected the number of bacteria and quality of the subgingival biofilm analyzed during the follow-up, leading to low DNA-DNA hybridization detection of the species of interest. It has been previously observed that professional supragingival cleaning of teeth can have an effect on gingival inflammation and subgingival bacterial composition; however, results are contradictory (Goodson et al., 2004; McNabb et al., 1992; Westfelt et al., 1998; Ximenez-Fyvie et al., 2000).

Finally, the detection of the assayed species was also evaluated according to tooth type sampled (Study IV, Figure 6). Most of the species assayed by whole genomic DNA probes were detected at higher levels from incisors of subjects exhibiting periodontal attachment loss over the observation time compared to inactive subjects (Study IV). Exemplifying this association, C. rectus from incisors and canines (p≤0.05) and E. saphenum from incisors (p≤0.05) were more frequently found in subjects with active attachment loss (Study IV, Figure 6). Both C. rectus and E. saphenum have been associated with periodontitis (Kumar et al., 2003; Moore & Moore, 1994).
Socio-demographic Factors in Early Periodontitis

In the cross-sectional analysis of the clinical data at baseline most participants were Caucasian (65%) and had never smoked (68%) (Study III, Table). A positive association between early periodontitis and the following socio-demographic variables was observed: older age (p=0.01), male gender (p=0.04), and cigarette smoking (p=0.05) (Study III, Table 1). These findings were no longer significant after the 18 month follow-up (Study IV, Table 1). A possible explanation could be due to bias by “loss to follow-up”, meaning that the subjects that stayed in the study and were observed over time were more uniform in their socio-demographic characteristics, compared to the subjects that did not continue to participate.

Aggregatibacter actinomycetemcomitans Lack of Serotype Antigen Expression (Study V)

In an attempt to study the role of bacterial subgroups in periodontitis, the serotypeability of clinical A. actinomycetemcomitans isolates recovered from various periodontal conditions was investigated (Study V). A. actinomycetemcomitans was detected in all populations studied in this thesis (Study I-IV) and because of its previously found association with periodontitis both in adults and younger subjects, the association of subgroups of this species with periodontitis was investigated.

Serotype identification using PCR in addition to immunodiffusion exhibited perfect concordance when immunodiffusion assay serotypeable strains were analyzed (Paper V, Table 2). Non-serotypeable strains (N=95) by immunodiffusion assay could be identified to serotypes a, b, c, or f by PCR analysis, showing that the strains carried serotype-specific genes (Study V, Table 3). Western blot analysis using respective antisera, antiserum against a non-serotypeable strain, and sera of patients with non-serotypeable A. actinomycetemcomitans strains confirmed the lack of expression of serotype-specific antigen in these strains (Study V, Figures 1, 2, and 3). Analyses of the serotype-specific gene cluster and sequencing of the whole serotype a gene cluster did not reveal any differences or any obvious DNA rearrangement between serotypeable and non-serotypeable strains.
It appeared that the non-serotypeable \textit{A. actinomycetemcomitans} strains did not include novel serotypes, but the reason for non-serotypeability was lack of expressing the serotype antigen. Most of these strains were detected more frequently from patients with chronic rather than aggressive forms of periodontitis. Furthermore, most of the strains belonged to a distinct genetic lineage (serotypes c) (Paper V, Table 3). The serotype-specific polysaccharide of \textit{A. actinomycetemcomitans} as the immunodominant antigen of the species (Page \textit{et al.}, 1991; Sims \textit{et al.}, 1991; Wilson \\& Schiferle, 1991) has been suggested to play a significant role in the co-aggregation of the species with other Gram-negative species and to mediate subgingival biofilm formation (Fujise \textit{et al.}, 2008; Kolenbrander \textit{et al.}, 1989; Rupani \textit{et al.}, 2008; Suzuki \textit{et al.}, 2006). Thus, lack of its expression would seem disadvantageous for the species, due to impaired biofilm formation. On the other hand, a benefit of the lack of serotype antigen expression could be a competitive advantage against the antibody-based host response over strains expressing this antigen. Whether these strains transiently or permanently express their lack of serotype expression was not investigated. It could be possible that the strains refrain from expression in certain phases of the biofilm formation or of host response.
Summary and Conclusions

A comprehensive assessment of children’s oral microbiota was achieved in this thesis through use of extensive molecular methods and examination of numerous children. In terms of species identification, 16S rDNA cloning and sequencing and whole genomic DNA probes complemented each other. Clonal analysis enabled an equitable identification of a diverse microbiota of 138 taxa, half of which have not been previously cultivated. In addition, 107 of 3909 clones remained unidentified and may include novel phylotypes. The DNA probe assay identified and quantified a selection of 74 cultured species. Out of these, 37 species were not detected by clonal analysis and certain species, such as *Actinomyces* species, were underrepresented.

The association of bacterial species with dental caries in children also differed according to molecular methods used. From the species detected by the DNA probe analysis of children’s dental plaque, 23% (17/74) were associated with childhood caries, including *Streptococcus*, *Actinomyces*, and *Lactobacillus* species, as well as certain periodontal pathogens. These findings confirmed and extended bacteriological findings regarding children's caries-related microbiota previously observed by molecular methods and culture. By clonal analysis, three out of the ten most prevalent species/phylotypes in children with caries were uncultivated or unnamed. Although clonal analysis might not be the optimal method for revealing associations of oral microbes with dental disease or health, the associations observed were clear and likely reflect the distribution of the predominant microbiota. Finally, the single-species PCR increased detection sensitivity of *S. mutans* present in low levels of the microbiota and enabled determining its association with dental caries in the children. Depending on the choice of the molecular method used, the microbiota of children can be approached from different viewpoints, focusing on the detection frequency of specific recognized pathogens, species quantification, and/or detection frequency of unnamed/uncultivable (even novel) phylotypes.

The microbiota of the early phase of adult periodontitis has been rarely studied, although incipient periodontal disease could be considered an informative model for studying initial microbial changes from health and during periodontitis progression. In this thesis, microbiological analysis of initial periodontitis was performed by
oligonucleotide and whole genomic DNA probes, and PCR. Generally, similarities were observed in the microbiota of initial periodontitis and previously well-characterized advanced periodontitis, with regards to detection of fastidious species such as *T. denticola*, *F. alocis*, and *P. endodontalis* and a strong association with *P. gingivalis* and *T. forsythia*. In addition, the detection of uncultivated *Treponema*, *Actinobaculum*, and *Bacteroidetes* phylotypes, as well as environmental species with no cultured representatives to date reveals the complexity of early adult periodontitis and warrants further investigation. Methodologically, the use of different molecular methods complemented each other also in the analysis of the microbiota associated with periodontitis. The PCR technique increased detection sensitivity of specific species and revealed their “hidden” associations with early periodontitis not observed with the other methods employed. The whole genomic DNA probes provided quantification of previously cultured species and the oligonucleotide DNA probes allowed screening of cultured and uncultivated phylotypes.

Besides bacterial diversity at species/phylotype level, different genotypes and phenotypes among strains of a species add complexity to the oral microbiota, as exemplified by the present study on *A. actinomycetemcomitans*. The reason for non-serotypeability of a number of *A. actinomycetemcomitans* strains was not the detection of novel serotypes or unrecognized clonal types as determined by genotyping, but lack of serotype antigen expression, despite that the strains carried serotype-specific genes. That the strains were mainly found in chronic periodontitis suggests that the serotype antigen deficient phenotype may provide a competitive advantage against humoral immune response over strains expressing this antigen.

Various molecular methods used in this thesis shed light on different subsets of oral bacterial populations, giving a variable perspective on their members. Until recently, identification of oral pathogens and their association with dental diseases has not been based on the microbiome of the oral cavity, but rather on the sporadic focus on specific species usually previously isolated by culture. Identification of the complete array of bacteria found in dental disease or health, through screening methods such as 16S rDNA cloning and sequencing, is pivotal and can in the future lead to identification of diagnostic bacterial profiles of dental diseases by more rapid molecular methods such as microarray analysis.
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