CHANGES IN THE ORAL MICROFLORA OF OCCLUSAL SURFACES OF TEETH WITH THE ONSET OF DENTAL CARIES

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Ágrip

Tannáta er mjög algengur sjúkdómur sem tengist samspili baktería í örveruþekkju "tannsýklu". Þrjár kenningar hafa verið notað til að lýsa þessum tengslunum á milli baktería og tannátu: (I) Sérhæfða kenningin „*specific plaque hypothesis"*, þar sem segir að fáar ákveðnar tegundir eru taldar valda tannátu; (II) Ósérhæfða kenningin „*non-specific plaque hypothesis"*, þar sem segir að heildarvirkni allra baktería í örveruþekkjunni valdi tannátu; (III) Vistfræðilega kenningin „*ecological plaque hypothesis"*, þar sem segir að breytingar á ríkjandi flóru séu vegna breytinga í vistkerfi, t.d. aukinni sýkursneyslu, sem svo leiði til aukins vaxtar á sýrumyndandi bakteríutegundum. Ræktun með sértæku (e. *selective*) æti er ágæt leið til að rannsaka sérhæfðu kenninguna. Aftur á móti er mjög erfitt að rannsaka breytingar í tannsýklu og heildar örveruflóru með hefðbundnum ræktunaraðferðum, þegar reynt er að kanna betur vitsfræðilegu kenninguna. Með tilkomu nákvæmra mælitækja sem geta metið heilbrigðar tennur og tannátu á byrjunarstigi á einfaldari hátt (*DIAGNOdent*®) ásamt því að nýta sameindlíffræðilegar aðferðir, gera okkur kleift að tengja betur staðbundnar breytingar á tannskemmdum við ákveðna bakteríuflóru.

Helstu markmið verkefnisins var að skoða bakteríuflóru í tannsýklu á nokkrum mismunandi stigum tannátu; (i) í tannsýklu ofan á glerung heilbrigðra tanna, (ii) tannsýklu úr glerungs-úrkölkun og (iii) úr byrjandi tannskemmdum. Áætlað var að bera að hluta niðurstöður klassískra aðferða með ræktunum saman við greiningu á örveruflóru með sameindalífræðilegum aðferðum, 16S rRNA gena greiningu og T-RFLP greiningu *(e. Terminal restriction fragment length polymorphism*). Báðar þessar aðferðir byggja á PCR mögnun á 16S rRNA geninu en nálgun aðferðanna er þó ólík. DNA röð þessa gens er vel þekkt hjá flestum tegundum baktería sem hafa verið ræktaðar og breytileiki í geninu gerir okkur kleift að greina á milli þeirra.

Helstu niðurstöður gefa til kynna að samsetning örveruflórunnar í tannsýklunni breytist mikið eftir því sem úrkölkun tanna ágerist. Mikinn örveru-fjölbreytileika var að finna í tannsýklu ofan á heilbrigðum glerung þar sem ýmsar *Streptococcus* tegunir voru algengastar. Fjölbreytileikinn minnkaði mikið við hækkandi sjúkdómsstig, aukna tannátu, þar sem sýrumyndandi og sýruþolandi örverur var að finna. Samanburður á milli sameindalíffræðilegra greininga og ræktunar sýndi aðallega fjölgun á *Lactobacillus* tegundum og öðrum sýruþolnum bakteríum með aukinni tannátu en bakteríur eins og *Actinomyces* sp. tókst ekki að rækta eða greina nema með sameindalíffræðilegum

aðferðum. Niðurstöðurnar í heild sýna ágætlega þær breytingar sem verða á tannsýklu á heilbrigðum glerungi yfir í tannátu á byrjunarstigi.

Abstract

Dental caries is one of the most common infectious diseases and is linked to bacteria in the dental plaque overlying the dental hard tissue. This link between plaque bacteria and the caries process has mainly been described by three hypotheses; (I) The *Specific plaque hypothesis*, which says only a very limited number of species are involved in the disease. (II) The *Non-specific plaque hypothesis* which says that all bacteria are equally effective in causing disease.(III) The *ecological plaque hypothesis* states that disease is due to a change in local environmental conditions, for example increased sugar consumption, which consequently disrupts the natural balance between plaque and the host, leading to increased growth of acid producing bacteria.

For studying the *specific plaque hypothesis*, cultivation of specific bacteria using selective media, is an excellent method. However, it can be difficult to investigate the changes in the dental plaque and identify the complete oral microflora, by traditional cultivation methods, when studying the *ecological plaque hypothesis*. With the development of the accurate and simple diagnostic equipment (*DIAGNOdent*®) it is possible to diagnose small differences between the healthy tooth surface and initial caries. This, in addition to the use of molecular methods for bacterial identification, allows investigation of the localized changes on the tooth surface that might be linked to certain bacterial flora.

The main aim of this project was to investigate the microflora of dental plaque samples from different stages of dental caries: (i) dental plaque on healthy tooth surface, (ii) dental plaque from initial caries, (iii) from early cavitated lesion and (iv) deep caries. Furthermore the aim was to compare the results of traditional cultivation methods with the results of bacterial identification by molecular methods, 16S rRNA gene analysis and T-RFLP analysis *(Terminal restriction fragment length polymorphism*). These two molecular methods are built on the amplification of the 16S rRNA gene but the methodological approach is slightly different. The sequence of the 16S rRNA gene is well known for most bacteria, is a well conserved sequence and the variations in the gene make it possible to distinguish between unrelated bacteria.

The main results showed that microbial composition in the dental plaque changed significantly with the progression of dental caries. Greatest microbial diversity was detected in the dental plaque on healthy tooth surface, dominated by various *Streptococcus*

species. Results from cultivation and molecular methods were consistent in showing increased growth of *Lactobacillus* sp. with increased caries. Other acid-tolerant bacteria and less acid-tolerant bacteria such as *Actinomyces* sp. could only be detected by molecular analysis but not cultivation. Results of this study demonstrated, in part, the reduced variety of the dental plaque microflora from the healthy tooth to initial caries and further into early cavitated lesions. The study adds to the increasing knowledge and understanding of the bacterial flora linked to dental caries.

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1. REVIEW OF LITERATURE

1.1 Introduction

It has been estimated that of the 10^{14} cells that make up the human body only 10% are mammalian. The remaining 90% are microorganisms that comprise the resident flora of the various surfaces of the human body. The human microflora, not least the oral microflora studied by van Leeuwenhoek in the 17th century, has been investigated extensively since the earliest days of microbiology and it is becoming increasingly clear that this microflora does not have a negative or passive relationship with the host, but contributes more or less directly and positively to host physiology, nutrition and defense systems. Although only a minor part of the human microflora is associated with diseases, pathogens have been of most interest to clinicians and researchers. Recently introduced culture-independent methods, such as 16S rRNA gene analysis, have altered the view of human microbial ecology greatly and the review paper by Dethlefsen and colleges, published in Nature 2007, illustrates the great diversity of the human microbiota (1). The oral cavity has been investigated increasingly using molecular methods during the last decade or so, especially in connection with identification of periodontal pathogens (2, 3) and now, more recently, the microflora of supra-gingival of healthy subjects (4) and dental caries (5-7). However, much of the current understanding of oral microbiology and of caries associated bacteria is based on the knowledge obtained from cultural-based studies. To understand fully how oral microorganisms persist, and under what circumstances cause diseases, it is necessary to have an understanding of the structure, function and biological activities of the oral microflora. Part of this understanding can be obtained by the detailed identification of oral microorganisms using molecular methods. The increasing data so obtained is altering, for example, the view of the pathological agents associated with dental caries. Nevertheless, more information of the caries-associated microflora is needed for further understanding of the caries process. One of the main reasons for the interest in the microbiology of initial caries is that, if the caries process is detected at earliest stages, the process can be stopped and partially reversed (8). This would consequently reduce the cost of treatments and the continued extension of caries which represents one of the most costly diseases that the majority of individuals will have to contend with in their lifetime (9). The literature concerning dental plaque and caries is vast and no effort

will be made in this thesis to cover the whole spectrum of previous research but will rather focus on the development of theories linking dental plaque flora with the carious process and the research methodology. It is this particular area that is relevant to the present studies.

1.2 Oral cavity - A habitat for diverse bacteria

From a microbial point of view, the oral cavity can be looked at as localized ecosystem; an ecosystem has been defined as a complex of organisms in a specified environment and the non-microbial surroundings with which the organisms are associated. The local environment of the oral cavity is rather small, with surface area around 215cm^2 (10) but its ecological characteristics are rather unique and complex. The unusual surfaces, such as lips, tongue, cheeks and the hard tissue of teeth, create number of varied habitats for the formation of mixed culture biofilms and with individual populations occupying different, and often highly specific, ecological niches. All these habitats differ with regard to surface and nutrient supplies. The biofilm covering tooth surfaces, termed the dental plaque, has been defined as a microbial community embedded in a matrix of polymers of bacterial and host origin. In addition to the habitat variation, there are numerous gradients in ecological factors inside the dental plaque, such as nutrient supplies, O_2/CO_2 levels, pH and redox potential. These factors are constantly changing, and thus provide both friendly and hostile environments suitable for microorganisms with a broad spectrum of requirements. Saliva also plays important role in regulating the metabolism and growth of oral bacteria (11). It helps maintain the pH around 6.25-7.25, the temperature at 35-36°C, optimal for the growth of many bacteria, and contains glycoprotein and proteins that act as the primary source of carbohydrates but also serves to dilute antimicrobial substances. The ecological conditions are constantly changing and, furthermore, properties of some of these habitats will change during the life of an individual. Changes such as from the primary to the permanent dentition, antibiotic therapy, tooth extraction, denture wearing and any other dental treatment, may affect the entire oral microbial community and make it even more complicated to identify and describe the oral microflora and the difference between health and disease.

Efforts to study and understand the mechanisms of the oral biofilm, have revealed that the formation and maturing of the microbial community is a highly specific process (12-16). Distinct stages in plaque formation include: acquired pellicle formation; reversible adhesion involving weak long-range physico-chemical interactions between the cell surface and the

pellicle, which can lead to stronger adhesin-receptor mediated attachment; co-adhesion resulting in attachment of secondary colonizers to already attached cells, multiplication and biofilm formation. Furthermore, the succession of the biofilm is a continuous process but the overall composition of the climax community of plaque is highly diverse. Overall, the bacterial community in the oral cavity is one of the most complex mixtures of bacteria known and the microbial profiles vary greatly from site to site (4). In total, more than 700 species have been identified, of which only 50% have been cultivated (2, 3). Most of these species constitute the normal flora of the oral cavity. However, under certain circumstances, changes in the environment cause the resident microflora to create a localized infection or even a systematic disease. These local infections in the oral cavity are of two main types, dental caries, the main focus of this thesis, and periodontal disease. Such diseases are the result of complex interactions between the resident microflora and the host. Dental caries has been described as a chronic disease which progresses slowly in most individuals. There is evidence that caries is not a classical infectious disease. Rather it results from an ecological shift in the tooth-surface biofilm leading to a mineral imbalance between plaque fluid and tooth and hence net loss of tooth mineral. Caries thus belongs to a group of common but 'complex' or 'multifactorial' diseases, such as cancer, cardiovascular diseases, diabetes, in which many genetic, environmental and behavioral risk factors interact (8). The disease is seldom self-limiting and, in the absence of treatment, caries may progress until the tooth is destroyed. Localized destruction of the hard tissue, often referred as the carious lesion, is the sign or symptom of the disease (17). Substantial pH fluctuations within the biofilm on the tooth surface are a ubiquitous and natural phenomenon, taking place at any time during the day and night. Regular removal of the biofilm, preferably with a toothpaste containing fluoride, delays or even arrests lesion progression and under the appropriate conditions this may lead to recalcification of early carious lesions. Reversal of caries can occur at any stage of lesion progression, because it is the biofilm at the tooth or cavity surface that drives the carious process (8).

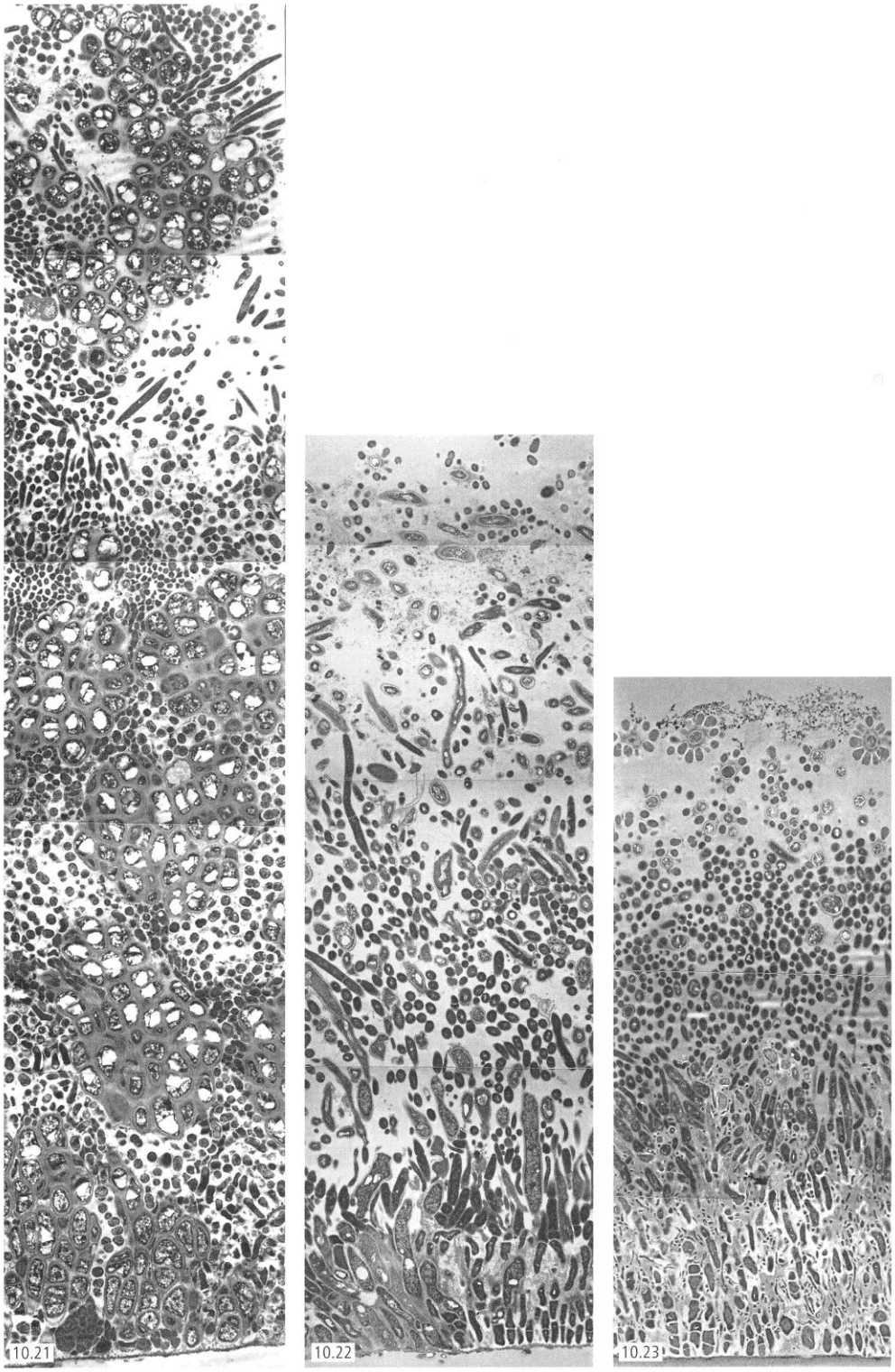

Figure 1. Ultrastructure of 2 week-old dental plaque from three individuals with different colonization patterns. In addition to difference in thickness, the outer part of the microbial deposits varies in composition and structure. Reproduced from Nyvad and Fejerskov (14) with permission from Blackwell-Munksgaard.

1.3 Brief historical review- pathogenesis of dental caries

Until the later part of the 20th century, oral microbiology was greatly influenced by the work of Robert Koch and his co-workers who developed simple methods for isolation of pure microbial cultures. The rapid advances in the search to identify the causes of infectious diseases linked to these studies of Koch and Pasteur who were developing the ”germ theory” of disease based on search for specific aetiological agents. Current knowledge of oral microbiology is mostly based on the knowledge from such pure cultures, based on studies over the past 100 years. The fundamentals of our understanding of the pathogenesis of dental caries date back to these studies. In 1890 WD Miller put forward his chemico-parasitic theory of tooth decay that today still holds true in its basic concept. Briefly, Miller suggested that bacteria accumulated on teeth and fermented dietary carbohydrate to produce lactic and other acids that then destroyed the tooth tissues. His search for the key pathogens in this process was less fruitful but, interestingly, Miller also realized that several potentially key pathogens were uncultivable in his hands. The key suspect bacteria would be those that produced acid and tolerated pH levels below that at which dental caries would occur. In 1924 Clarke discovered *Streptococcus mutans* in carious lesions and this later became the organism most suspected of causing caries although at the time lactobacilli were more favored as the key cariogenic pathogens due to their considerable tolerance of low pH values. By the mid 1950’s, Orland and co-workers began to study caries using an animal model system with gnotobiotic hamsters that were fed a diet rich in refined carbohydrate and were infected initially with enterococci, but substituted by *S. mutans* in later studies (18). Studies with this type of animal model allowed several candidates of cariogenic bacteria to be tested but *S. mutans* soon became established as the key pathogen. In the same study lactobacilli were not able to initiate lesions, possibly because they failed to adhere to the enamel, but could effectively decalcify tooth tissue that had already become partly decayed.

Much of the belief in *S. mutans* specificity as a caries pathogen is built up from these experimental animal studies (18) which were of such significance that this bacterial species is still considered the most specific of the bacteria that cause caries. Those studies have led to findings that *S. mutans* have the ability to ferment sucrose and induce caries formation in animals when fed a sucrose-rich diet. More reasons for the emphasis on *S. mutans* as the prime cariogenic organisms are discussed by Takahashi and Nyvad 2008 (19). These cariogenic factors include

frequent isolation from cavitated caries lesions, acid tolerance, acid production and the production of large amounts of extracellular sticky glucans that aid bacterial adhesion to teeth (19). As well as studies with experimental models and clinical samples to demonstrate the association of mutans streptococci with caries, other researchers have used counts of these bacteria and also lactobacilli to estimate caries risk and to monitor the success of preventive measures (20-23). Perhaps the "high point" of consideration of the role of *S. mutans* in dental caries was reached with the various attempts to immunize patients against this organism and its disease with what has been considered as the "caries vaccine" (24-26).

While the role of bacteria in causing tooth decay is quite clear, there is a marked difference between a bacterial cause of caries and the usual types of bacterial infection, caused most commonly by an exogenous organism such as with Streptococcal sore throat or an endogenous organism moving to a new site e.g. infective endocarditis, that is frequently caused by oral streptococci. As mentioned above, dental caries is an unusual form of infection by one or, more likely, a combination of organisms that are essentially commensal in the mouth. The role of dental plaque bacteria in causing this infection has been described in three rather differing hypotheses: the non-specific plaque hypothesis (27), the specific plaque hypothesis (28) and the ecological plaque hypothesis (29). Firstly, the specific plaque hypothesis is built on knowledge from culture-based studies and claims that only a few specific bacteria could affect tooth enamel. There have been many studies and publications on the correlation between caries and, particularly, the number of mutans streptococci and oral lactobacilli. Such bacterial counts have been demonstrated as an accurate indicator of dental caries-activity and used as tests of caries activity (23). However, new advanced culture-independent methods have shown that the dental plaque biofilm is far more complex in bacterial composition and structure than had been assumed. A high proportion of mutans streptococci may persist on tooth surface without lesion development, and caries can develop in the absence of these species (5, 30). Under such circumstances, it is suggested that other acidogenic bacteria as well as *S. mutans* , including low pH non-mutans streptococci and *Actinomyces* species are responsible for the initiation of caries (19). Recent molecular studies have strengthened this concept by showing that the microflora associated with white spot, or initial carious, lesions is more diverse than previously predicted

and includes novel phylotypes and species such as *Actinomyces naeslundii, Actinomyces israelii*, a broad range of non- mutans *Streptococcus* sp. and *Veillonella* sp. (5, 6).

Thus new investigations have suggested a move from the *specific plaque hypothesis* of Loesche (28) that implicated specific bacteria in caries aetiology towards the *ecological plaque hypothesis* proposed by Marsh and co-workers (29) where the view is held that organisms in the complex biofilm can work together or in conflict depending on the environmental circumstances. Key features of this hypothesis are that (a) the selection of "pathogenic" bacteria is directly coupled to changes in the environment and (b) diseases need not have a specific etiology; any species with relevant traits can contribute to the disease process. Thus, mutans streptococci are among the best adapted organisms to the cariogenic environment (high sugar/low pH), but such traits are not unique to these bacteria (31). Briefly, the biofilm would respond to a diet rich in fermentable carbohydrate by allowing acid-producing and acid-tolerant organisms (e.g. mutans streptococci, *Lactobacillus* sp. and others) to multiply. Bacteria, such as *Veillonella* sp. that can utilize lactic acid would help in preventing inhibition of mutans streptococci when the pH dropped below the optimum for that group of species (32). The net result would be such that the biofilm would develop in the direction of acid production and tolerance of low pH while, at the same time, dextran production by biofilm bacteria would adhere the biofilm better to the teeth. Moreover, the dextran-glycoprotein plaque matrix would become thicker, further inhibiting the acid-buffering effect of saliva on the tooth surface. A major consequence of this would be that dental enamel, composed largely of hydroxyl-apatite crystals, would begin to decalcify and the crystals would disintegrate under the acid attack and the disease, known as dental caries, would be initiated. Together with changes in the explanation of the microbial etiology of caries, novel

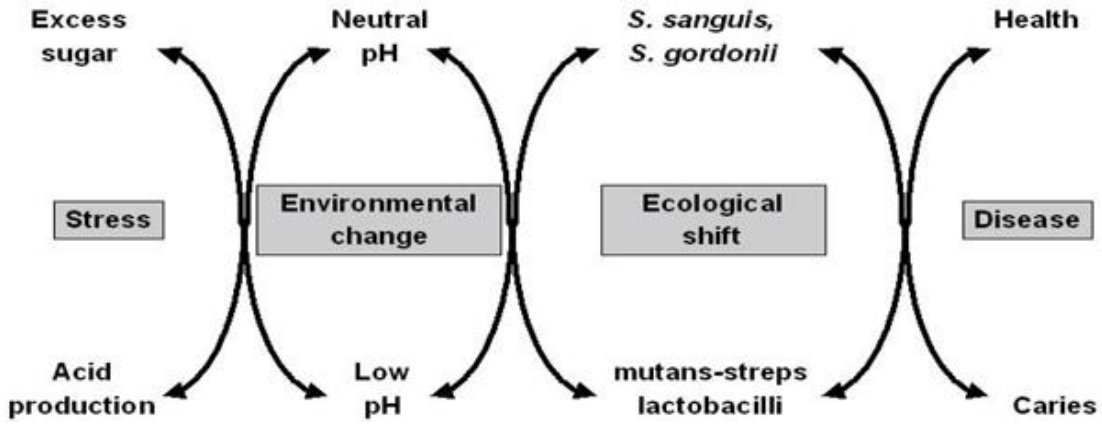

Figure 2. A schematic representation of ecological plaque hypothesis in the relation to the aetiology of dental caries (29).

concepts have evolved around the carious process itself. Thus, there is a growing awareness that caries lesions, under certain circumstances, can be managed by non-operative intervention (17).

The specific plaque hypothesis has been considerably modified with regard to periodontal disease and now numerous bacteria in collaboration are thought to play important roles in promoting that dental plaque-related disease. With respect to dental caries, however, there has been a dominance of a specific plaque hypothesis involving two major species of mutans streptococci, *S. mutans* and *Streptococcus sobrinus,* as the chief pathogens initiating caries. The initial lesion could then be continued by other acid-tolerant bacteria such as lactobacilli and *Actinomyces* sp. With the proposal of the *ecological plaque hypothesis* this view of the predominance of mutans streptococci and lactobacilli as the aetiological agents of caries is beginning to be revised. This study aimed to investigate the changes occurring in the dental plaque biofilm with the change from healthy tooth surfaces to dental caries.

1.4 Assessing microbial diversity of oral bacteria

One area within the practice of clinical microbiology is the craft of putting scientific names to, and identification of, microbial isolates. The historical method for performing this task is dependent on the comparison of an accurate morphologic and phenotypic description of type strains or typical strains with the accurate morphologic and phenotypic description of the isolate to be identified. Accurate identification and quantification of complex mixtures of bacteria such as those found in the oral cavity are difficult to process.

1.4.1 Sampling strategies

Firstly the sampling procedure must ensure that numbers and proportions of microorganisms in the sample are not altered, either positively or negatively, in a non-quantifiable manner during collection or storage of the sample. Sampling procedures must also ensure that samples are representative for the microbial community and are not contaminated with foreign microorganisms. The microflora can vary in composition over a relatively small distance. Therefore, large plaque samples or the pooling of smaller samples from different sites, are of little value because important site difference will be obscured (9). Several sampling strategies for oral microbes have been developed to access bacteria from the various sites in the mouth. Salvia is easily sampled, but it contains a mix of bacteria shed from many ecosystems, sub- and the

supragingival, teeth, tongue, cheeks etc. One approach is to use a curette to scrape the biofilm off the tooth, most commonly used for supragingival plaque. The alternative approach is to use a paper point that consists of very thin paper rolled into a stick approximately 1.5-2 mm in diameter and 3 cm long. A broad spectrum of bacteria can be recovered and detected by using this approach for sampling (3).

1.4.2 Cultivation of oral bacteria

Studies have attempted to look at the total viable count of the dental plaque microflora, following aerobic and anaerobic culture of samples on various selective and non-selective media. This type of investigation is extremely difficult to complete satisfactorily as samples are difficult to disperse, to transport without loss of viability, to culture, quantify and to identify. It is obvious, that conditions not compatible with the physiological requirements of particular group of microorganisms will lead to their underestimation because of loss of viability. The detection of microorganisms based on phenotypic characters requires that the microorganism to be detected must be recovered by culture from environmental sample, such as the oral cavity. The classical approach for detecting microorganisms is to place viable cells onto a solid medium or into a liquid broth containing all the nutrients essential for the growth of the target microorganism, and incubate cultures under conditions that favor the growth of those microbes so their phenotypes can be observed. Cultivation on broad-spectrum, non-selective media such as blood agar supports the growth of many oral species. An oral sample typically produces a diverse array of colony morphologies so it can difficult to sort out individual species from the mix, and species that compromise only small percentage of total bacteria may not even be seen. Continued investigation to complete the identification of the phenotypes can be done using a set of selective media and physical tests (33).

For clinical use a few selective media for oral bacteria have been used to enable culture of lactobacilli (34); for *S. mutans* , mitis-salivarius-bacitracin agar (35) and for *S. sobrinus*, tryptic-soy yeast extract-bacitracin agar. Clinical samples are transported in relatively stable media such as reduced transport fluid or VMG II (36), dispersed and inoculated on to the highly selective media. Identification is relatively straightforward as these media are highly- selective and counts are possible. However, studies have shown that the counts on selective media are lower than when other means of identification are used (such as monoclonal antibody staining)

(37). The advantages of such selective media are that relevant information has been obtainable relating bacterial counts to caries status and the risk of future caries (so-called caries activity testing) (38-40). However, culture methods do not do much to help explain the carious process and many researches are increasingly demonstrating that the microflora involved is more complicated (5, 12, 26, 29, 31, 41-43). Other methods were, consequently, needed to help explain the association of bacteria within the dental plaque biofilm with the onset of caries. Additionally, as mentioned before, cultivation does not give the full picture of the diversity of the complex oral bacterial community. Thus, the concepts about dental diseases are based upon microbial knowledge from only a part of the oral bacterial population and are biased towards those parts which are cultivable under laboratory conditions.

1.4.3 Molecular method-for identification and classification of oral bacteria

The rapid development methods based on PCR amplification have opened a new window for investigation of the microbial world. There are two issues of profound importance; first the discovery of phylogenic informative DNA sequence such as 16S rRNA gene. These discoveries have changed the concept of bacterial relatedness and provided a universal system for identification and categorization. Second, it became possible to detect and identify unknown and uncultivable bacteria and study the diversity and spatial organization of complex microbial communities. As mentioned above, our understanding of oral microbiology and oral infectious diseases is mostly obtained from culture-dependent methods. These methods have provided a good indication of the microflora involved in the caries demineralization process and are still good for identification of known pathogens and other cultivable oral bacteria.

Classification is the arrangement of organisms into groups (taxa) on the basis of their similarities and differences. Traditionally, a hierarchical system has existed for naming of bacteria so that groups of closely related organisms form species and related species are placed in genus, families, etc. This requires the determination and comparison of as many characteristics as possible, though in identification scheme, only a few key discriminatory tests may be needed.

In a paper by Zuckerkandl and Pauline, (1965), it is well-defined how it would be possible to use proteins and DNA as molecular chronometers to compare evolutionary relations between organisms (44). Before that microbiologists had to rely on their ability to recognize bacteria by phenotypic characters using visual examination, microscopic appearances, size,

individual colony and color, growth conditions and other physical and biochemical testing to distinguish between species. These characteristics are, however, too often subjective especially when the organism is as small as a bacterium and there is not much room for morphological diversity at the level of unicellular organisms (45). The work of Carl Woese and colleges with the attempt to use rRNA directly to characterize microbes from environmental samples was a breakthrough formulation for microbial research. This work provided an objective framework for identification and also determining the evolutionary relationship between organisms. In the paper Bacterial Evolution, Woese (46) illustrated in detail the measurements of bacterial phylogeny and what is gained using proteins and nucleic acids as an evolutionary clock. It is also well demonstrated what specifications such molecules need to meet in order to be useful as evolutionary chronometers. That includes (i) clocklike behavior (changes in its sequence have to occur as randomly as possible), (ii) phylogenic range (rates of change have to be proportionate with the spectrum of evolutionary distance being measured), and (iii) size and accuracy (the molecule has to be large enough to provide sufficient amount of information (46). The 16S rRNA molecule meets these criteria quite well. This shows a high degree of functional consistency, has long highly-conserved regions which are useful for measuring distant phylogenic relationships and sufficient variable regions to assess a close relationship. It occurs in all microorganisms and the gene is large, about 50 helices in the 16S rRNA secondary structure, and they consist of many domains. Additionally, and importantly, 16S rRNA is still small enough to be isolated and amplified and sequenced directly. Although the use of molecular methods alone is not sufficient for the classification of certain microorganism, the advent of molecular approaches to classification has allowed bacteria to be grouped according to their "natural" relationship, thereby showing their evolutionary relationship as phylogenic trees. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (47). Based on such sequence analyses of ribosomal RNA homologies, a detailed evolutionary tree has been developed. This evolutionary tree of life, indicates that three evolutionary lines (domains) diverge from common ancestral organism to form the Archeaebacteria, Eubacteria and Eucarya (48).

1.4.4 16S rRNA gene sequencing analysis

The amplification of template DNA of interest or a mixture of homologous genes, as the 16S rRNA gene, is today most commonly used in microbial ecology studies. The practical approach for 16S rRNA gene analysis for community analysis is the building of clone-libraries from amplified DNA. The DNA of the community sample is made accessible by traditional cell phenol-chloroform lysis method or by any of the various commercial methods that have been described. Although the 16S rRNA gene is around 1500 nucleotides, the initial 500 bases of the gene provide adequate phylogenic information for identification (49). On a practical note, generating the 500-bp sequence is less expensive and easier as it takes more sequencing reactions to generate the 1500-bp sequence. 16S rRNA gene or gene fragment can be selectively amplified by PCR using oligo-nucleotide probes (primers). "Universal" primer sets are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (50), or targeted at specific taxonomic level, phylum, families, genus etc.. Other areas of the rRNA gene have also been used for studying phylogenic relationships among bacteria. Roth et al. (51) used the 16S-23S rRNA gene internal transcribed spacer sequences to distinguish among *Mycobacterium* spp., finding it particularly useful for species that were indistinguishable by 16S rRNA gene sequences. Furthermore, if the interest is only in differentiating species within a particular genus, a better gene than the 16S rRNA gene might be found to identify species. However, no gene has shown such broad applicability over all the taxonomic groups as the 16S rRNA gene. Thus, if the goal is to identify a broad spectrum of bacteria or even unknown microorganisms, the 16S rRNA gene sequence is an excellent and extensively used choice (49). After the amplification, PCR products produced are purified, ligated into a plasmid vector and then *E. coli* is transfected with the plasmid DNA. With this procedure the diverse 16S rRNA genes are separated and clone libraries (generally around 100 up to 1000 clones) are built. The inserted 16S rDNA is amplified from randomly selected clones and sequenced (usually 300-1000 bases) and analyzed further. Ideally, the clone library covers the entire population of rRNA molecules from the microbial community under investigation and there for a good indication of the real microbial diversity can be obtained. The subsequent analysis is to compare the 16S rRNA sequences with previously

identified once in the large accessible databases, such as GeneBank (http://www.ncbi.nlm.nih.gov/) and the Ribosomal database project (RDP-II) (http://rdp.cme.msu.edu/html/). The final input of 16S rRNA analysis is the graphical creation of phylogenic trees, for the interpretation of the data. Comparisons are commonly shown as dendrograms and linear alignments, for concise linear alignments, all the identical base pairs are omitted and only the differences are shown. One method most commonly used for generating dendrograms is the NJ (neighbor-joining) method (52). The dendrogram is also beneficial to assess an unknown sequence were the sequence classified to its closest known relative. This 16S rRNA methodology can be applied to virtually any environment and enables us to discriminate between organisms to the same genus or even between species, although not between strain within the same species (45).

It is important to note that if using online public databases, such as GeneBank and Ribosomal Database, for gene sequences comparison of 16S rRNA sequences, not all cultivable bacteria has been deposited into the database. Thus, not matching specific clone sequences to cultivable representative sequence, does not mean is has not been cultured. Additionally, despite the fact that the advent of molecular technique has allowed the identification of new still uncultivated species the cultivation techniques are needed to move ahead with these yet-to-be cultivated species.

1.4.5 Terminal Restriction Fragment Length Polymorphism -T-RFLP

The terminal-restriction fragment length polymorphism method was introduced at the end of 1990s (53). The T-RFLP method, as the name indicates, is based on discrimination of unrelated bacteria on different length of terminal fragments of an amplified DNA digested by restriction enzymes. Amplification is performed using primers, usually universal for the 16rRNA gene, with fluorescent molecules attached to them. This allows the detection of the terminal end of the digested DNA. Various restriction enzymes can be used, but they catalyze cleavage of double-stranded DNA through the recognition of specific nucleotide sequence. The location of these restriction sites on the 16S rRNA gene are not random, but have phylogenic relations, therefore the method can provide some taxonomic knowledge for the community under investigation. The diversity of community sample is in some cases underestimated (54) where the 16S rRNA gene of closely-related bacteria can have the same terminal restriction sites for some of the same

restriction enzymes. There are still only a few studies reported using this method in oral microbiology but the method has been used for community profiling and as well for monitoring changes in many other ecological studies (55, 56). Sakamoto and his colleges showed that T-RFLP analysis for the estimation of diversity of bacterial oral flora in patients with and without periodontitis and for comparison between subjects and community structure (57). This method has been shown to be highly effective to study changes in various microbial communities and the differences between samples from similar environment. Furthermore, the method has the advantage of being inexpensive and especially robust; hundreds of reproducible TRF patterns can be generated in a short time. This means that it is possible to determine spatial and temporal shifts in community structure with some confidence. The advantages and disadvantages of the method have been evaluated in at least two separate review papers (54, 58).

Other culture-independent methods, such as *denaturing gradient gel electrophoresis* (DGGE) and *fluorescence in-situ hybridization (*FISH) and several other methods have also been used for analysis of oral microbiota. One method, introduced by Socransky and co-workers (1994), is the Checkerboard DNA-DNA hybridization method that is frequently used in studies of the oral microflora (12, 59, 60) This is a more selective method not suitable for "not-yet-cultured" bacteria, and only detecting the species of corresponding probes of the panel used for the hybridization but, on the other hand, it is more quantitative.

1.5 Pitfalls and biases produced by culture-independent methods

Culture-independent methods for microbial studies are revolutionary, equally to show the evolutionary relation between species, to identify and describe bacterial diversity in complex environments as the oral cavity (4, 61) and as well to reach the unknown and uncultivable microorganisms (62). The large, and still growing, amount of 16S rRNA sequence data available in large public databanks on the internet is also important. However, cultivation-independent methods have their flaws and biases and many of these pitfalls have been demonstrated in a number of studies and reviews (63-65). These methods are based on many physical, chemical and biological steps and each and every of them can produce biases, that lead to results that do not reflect the true microbial composition. Sampling and DNA extraction are often ignored as a source of problems in microbial studies, but are actually crucial for all subsequent analyses. Due

to differences in the susceptibility of bacterial cells to lysis, the methods optimal for DNA extraction from Gram-negative bacteria, may extract insufficient amount of DNA from Gram-positive bacteria. It has to be considered that PCR methods are extremely sensitive and only small amount of DNA is needed for amplification, thus a special care must be taken to avoid contamination, especially during their transport, storage and processing (64). Amplified DNA can only reflect the quantitative abundance of species in a sample if the amplification efficiency is the same for all DNA templates. However, in such multi-template PCR, the natural variation in the target genes makes it almost impossible to avoid biases completely. The primer sets are never absolutely universal, leading to unequal hybridization to target DNA and, furthermore, amplification of 16S rRNA favors bacteria with a higher copy number of rRNA gene operons in their genomes. Additional biases may arise from PCR-amplification inhibitors, degradation of DNA or the use of a different cloning system (64). All these factors may have an effect on the identification and quantification of different bacteria in the community samples. To obtain reliable data from such PCR-based analysis, reduction of biases and artifacts is essential. However, by being aware of these pitfalls, some of them can be minimized and even avoided completely.

1.6 Diagnosis of dental caries

Dental decay is measured clinically as a cavitation on the tooth surface. However, cavitation is a late event in the pathogenesis of decay, having been preceded by a clinically detectable subsurface lesion known as a white spot and prior to that by subsurface demineralization that can only be detected microscopically. From a diagnostic and treatment perspective, the lesion should be detected at the early decalcification or white-spot stage. This usually cannot be done without rigorous descriptive criteria and because the white spot stage in the caries-prone fissures and approximal surfaces cannot be directly visualized during a routine dental examination. Thus, in some of the bacteriological studies which have been performed, there is the possibility that the flora being associated with the decay is the result, not the cause, of the cavitation (28).

While beyond the main aim of this thesis it should be pointed out that teeth have a definite but somewhat limited capacity to recalcify, thus reversing the carious process (8). These results from, plaque removal, thorough oral hygiene measures and topical fluoride applications that mostly work by promoting recalcification of decalcified, but not destroyed,

enamel crystals. Fluoride is also, in high concentrations, antibacterial but this is thought to play only a minor role in caries prevention or reversal as the high levels of fluoride are soon diluted by saliva. Once the tooth enamel has become initially decalcified in the carious process, a succession of microorganisms in the dental plaque biofilm appears to be able to continue the process leading to cavitations and subsequent extension of the lesion. These findings make it important to use accurate diagnostic equipment to increase our knowledge of the bacterial flora involved in the initial stage of caries development in order to help prevent or even reverse the carious process.

Dental caries lesions were formerly diagnosed at the stage of cavitation by using a dental probe ("explorer"). However, the exploration with sharp instrument not only damages weakened tooth structure that may otherwise remineralize, but also could inoculate non-carious fissure with cariogenic bacteria (66). When it was realized that this could be damaging to repairable tooth decay, more visual methods for diagnosing caries have been adopted (67) but with varying degrees of success, especially if the teeth were not free of overlying plaque biofilm. Consequently there was a need for the development of sensitive diagnostic tools such as the fluorescent device, DIAGNOdent®, that could detect early decalcifications at a stage where recalcification was possible (68). While it is not completely clear what, precisely, the DIAGNOdent equipment is measuring the increasing score of detected red fluorescence, presumed to be from porphyrins, has been shown to correlate reasonably well with developing caries lesions, at least sufficiently well to classify occlusal surfaces of molar and premolar teeth as healthy, with initial disease and with established cavitation, albeit cavitation that is hardly visible to the naked eye. Obvious and large cavities were not included in this study as it proved impossible to get DIAGNOdent readings greater than those recorded for the small cavities.

2. AIM OF THE PRESENT STUDY

The aim of this study was to investigate the dominant microflora of occlusal tooth surfaces at different stages from health to early dental caries in order to determine the possible role of specific bacteria in the carious process or to observe changes in diversity that might occur before or after the increased disease state. The health/disease status of the tooth surface was determined as accurately as possible by visual examination and using the DIAGNOdent equipment. Standard cultural methods and culture-independent techniques were used and compared for assessing the composition of the dental plaque biofilm overlying the respective tooth surfaces being investigated. The expected outcome of this investigation will add information relevant to the continuing debate over the role of specific or non-specific microorganisms in the development of dental caries.

3. MATERIALS AND METHODS

3.1 Subjects

Participants in this study were patients or student volunteers in Faculty of Odontology, University of Iceland. All gave their informed consent to the sensitive assessment of occlusal caries and collection of a plaque sample. Samples were all rendered anonymous immediately after collection and only the degree of caries was noted when the sample was transferred to the research laboratory. Specimens required for caries activity testing from some of the test subjects were collected immediately following the test sample and were handled routinely in the diagnostic laboratory of the Faculty of Odontology.

3.2 Caries grading and sampling

Samples were collected by a single dentist (WPH) from the occlusal surfaces of molar and/ or premolar teeth. First the degree of caries was estimated using the DIAGNOdent® fluorescent detection apparatus (KaVo, Biberach, Germany). This equipment is now marketed as a diagnostic aid for caries diagnosis but its accuracy and precisely what factor is measured by the DIAGNOdent® apparatus are not yet clear but the equipments being investigated and assessed by Álfheiður Ástvaldsdóttir as part of her doctoral studies. The DIAGNOdent® apparatus detects decalcification by emitting a red light of 650nm wavelength. This generates fluorescent light that is reflected back to the detecting probe and recorded by the equipment on a scale of 0-99. While the equipment may still be controversial (68) with respect to what is actually measured, the

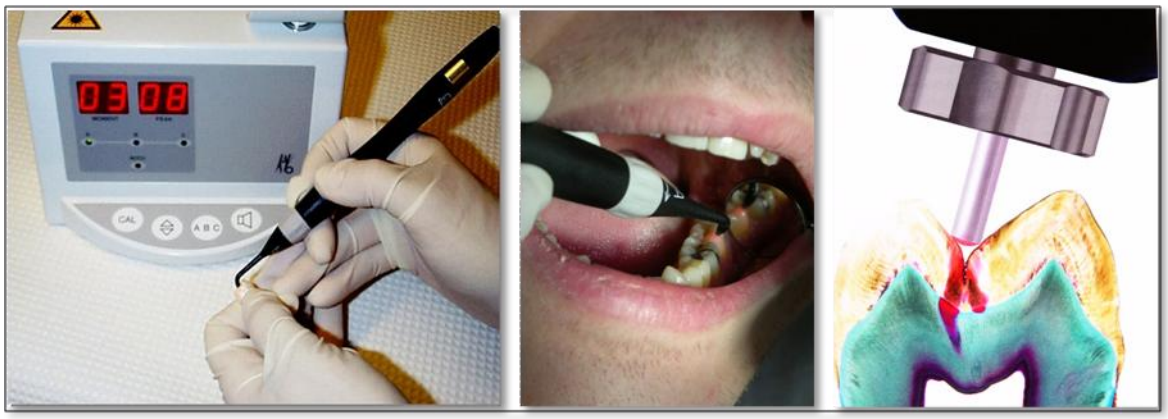

Figure 2. The degree of caries was estimated by visual examination and by the DIAGNOdent® fluorescent detection apparatus**.**

equipment is thought to give a reasonable estimate of the degree of occlusal caries present. One of the chief confounding factors to a DIAGNOdent® reading comes from false positive readings from dental calculus and tooth stain. These confounders were avoided as much as possible in sampling from the test subjects.

Before sampling all teeth were examined visually, evaluated and categorized. Molar and premolar teeth were also measured with the DIAGNOdent® fluorescent detection apparatus (KaVo, Biberach, Germany). Thus visual and DIAGNOdent® assessments were combined in order to diagnose the stage of tooth decay and to standardize the types of samples collected for further analysis.

The aim was to take 4 to 5 samples from four various stages of the caries lesion: (i) healthy, (ii) initial caries, (iii) early cavitated caries and (iv) open and deep cavitated caries. Thirteen good samples from three of these stages were obtained, but repeated sampling from deeper cavitated caries (stage iv) did not work out as expected. No colonies were recovered from cultures nor were any PCR products obtained from these samples and therefore this stage was not included in the further analysis. Samples are listed in Table 1 and categorized.

Table 1. Overview of categorization of analyzed samples

Subject	Sample ID	Clinical diagnosis
1	CF1	caries free
2	CF2	caries free
3	CF3	caries free
4	CF4	caries free
5	CF5	caries free
6	CB1	initial caries
7	CB2	initial caries
8	CB3	initial caries
9	CB4	initial caries
10	CA1	early cavitation
11	CA2	early cavitation
12	CA3	early cavitation
13	CA4	early cavitation

Samples were taken from patients with a DIAGNOdent® reading of 0-20 and these were deemed to be caries free. Other samples were taken from teeth where the DIAGNOdent® reading was 25-60 and these were categorized as initial caries. The third group of samples came

from teeth with lesions that registered 99 using the DIAGNOdent® equipment and classified as early cavitation. A final group of samples was taken from lesions that were visually extensively cavitated where the floor of the lesion was softened dentine. Samples were collected by inserting a sterile paper point, such as is used routinely in endodontics, into the occlusal fissure of teeth that had previously been assessed by the DIAGNOdent® apparatus. The paper point was removed after 5-10 seconds and placed into 1.5 ml eppendorf tubes containing 200µl of sterile reduced transport fluid, RTF (36) and directly transported to laboratory and kept at 4°C until processed further. When the cavity was large, paper points were pushed on to the softened dentine using sterile tweezers and then placed in the transport fluid.

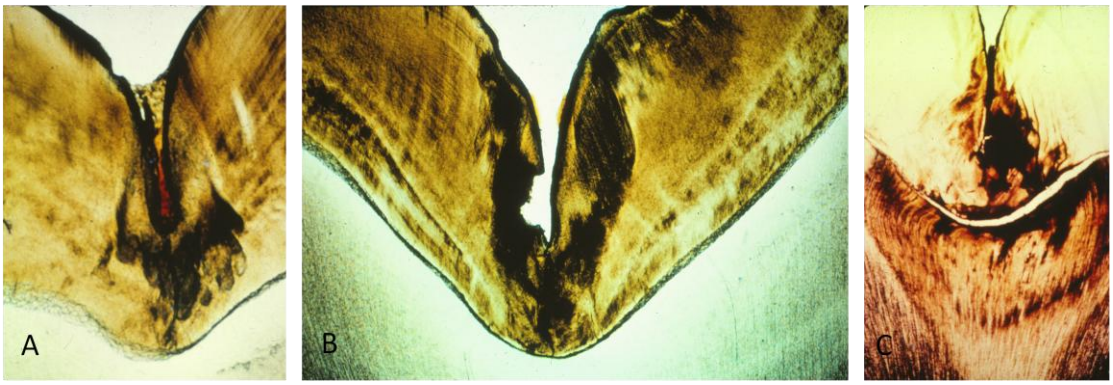

Figure 3. A.Ground sections of a tooth showing initial (enamel) caries in an occlusal fissure. B- Ground section showing initial(enamel) caries in an occlusal fissure, more advanced than in picture A. C- Ground section showing fissure caries that is extending into dentine (early cavitation)

3.3 Bacterial cultures

Of the 200µl sample, 50µl were used for culture on agar plates, the remaining 150µl were centrifuged at 5000 rpm and kept at -20°C until processed for DNA isolation. Bacterial cultures were performed to estimate viable bacterial counts of samples. Aliquot samples of 50µL of 10x and 100x dilutions were spread over the surface of specific and non-specific culture media using a plate rotator (Denley Scientific, Colchester, UK). Blood agar was used to determine total bacterial count under aerobic and anaerobic conditions. Rogosa agar (Difco) was used for cultivation of *Lactobacillus spp* and mitis-salivarius bacitracin agar (Difco) (35) for counts of *S. mutans* . Blood agar plates for total aerobic counts and MSB plates for *S. mutans* were placed in a candle jar and incubated at 37°C for 48 hours. Rogosa plates for *Lactobacillus* sp. culture were first covered with a thin layer of additional Rogosa agar and incubated aerobically at 37° for 48 hours as is the routine practice in the service laboratory. Blood agar plates for total anaerobic

count were incubated at 37°C in under anaerobic conditions using an anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) with a standard gas mixture of N_2, H_2 and CO_2 for one week. Colonies were evaluated and counted using a plate counter (Don Whitley Scientific).

3.4 Cell lysis and DNA extraction

To obtain DNA for molecular analysis, sample lyses were performed using method modified from Paster et al. (2001) (3). A 50µl solution containing Tris-HCl EDTA-buffer pH 7.8, 0.5% final concentration of Tween 20 and proteinase K (200µg/ml final conc.) were added to the samples. The samples were heated to 55°C for two hours in a water-bath and mixed carefully every 30 minutes. To inactivate the proteinase K, samples were heated to 95°C for 5 minutes. Then the eppendorf tubes containing the samples were placed on ice or in -20°C for further processing.

3.5 16S rRNA gene amplification

Amplification of 16S rRNA genes were performed under standard conditions, using universal selective primers F.27, 9.F, 1544.R and 805.R (69) for detection of Bacteria, depending on the amplification efficiency and success. 16S rRNA primers 23FLP and 1391R were used for detection of Archaea (69).

Table 2. Primers used for the amplification of 16S rRNA genes and sequencing

Primer	Sequence	
F.9	5′-GAGTTTGATYMTGGCTCAG-3′	Bacteria selective
F.27	5′-AGAGTTTGATCCTGGCTCAG-3′	Bacteria selective
R.1544	5′-AGAAAGGAGGTGATCCA-3′	Bacteria selective
R.805	5′-GACTACCCGGGTATCTAATCC-3′	Bacteria selective
FPL23	5'-GCGGATCCGCGGCCGCTGCAGAYCTGGTYGATYCTGCC-3'	Archaea selective
R1391	5'-GACGGGCGGTGTGTRCA-3'	Archaea selective
M13F	5´-GTAAAACGACGGCCAG-3´	Sequencing primer
M13R	5´-CAGGAAACAGCTATGAC-3´	Sequencing primer

PCR amplification for 16S rRNA was performed in 40µl volume of the following reagents: 30.3µl of sterile dH_2O, 1.0µ dNTP (10mM), 4,0µl 10x Buffer, 1,0µl Forward primer (20µM), 1.0µl Reverse primer (20µM) 0.7µl Teg polymerase and 2,0 µl of 10x fold dilutions of DNA template. The PCR protocol was the following: 94°C for 5 minutes followed by 34 cycles of

94°C for 30 seconds, annealing temperature ranging from 45°C-55°C for 50 sec each cycle with extension temperature at 72°C for 90 sec. with final extension period for 10 min at 72°C.

3.6 Electrophoresis

All 16S rRNA PCR products were examined with electrophoresis on 1% agarose gel stained with ethidium bromide and visualized under UV-light. To identify the size of the PCR product, which is around 1500 nucleotides, the PCR product was compared to a 1Kb ladder. Products of expected size were cut from the agarose gel and purified.

3.7 Purification of PCR products

PCR products were cut from the agarose gel with a sterile razorblade as close to the DNA band as possible and the slice placed in a sterile 1.5ml eppendorf tubes and purified using GFX purification kit (GE Healthcare Europe GmbH, Hillerød, Denmark). Approximately 10µl of capture buffer was used for each 10mg of gel slice. The tube was mixed by vortexing vigorously and incubated at 60°C for 5-15 min. or until the agarose gel was completely dissolved. Dissolved agarose gel was centrifuged briefly to collect the sample in the bottom of the eppendorf tube. Then dissolved sample was transferred into a GFX column in a collection tube and incubated at room temperature for 1 minute. The sample was centrifuged for 30 sec. at full speed (13.000 rpm). The supernatant was discarded from the collection tube and the column put back inside the tube. For each sample a 500µl of wash-buffer were put into the column and centrifuged at full speed. The GFX column was transferred into a new eppendorf tube and 30µl of autoclaved double-distilled water was applied directly on the top of glass fiber matrix in the GFX column. The sample was incubated at room temperature for 1 minute and centrifuged at full speed for 1 minute to recover the DNA.

3.8 Cloning procedure

To increase the efficiency in the cloning procedure, A-nucleotides were added to the ends of the PCR product before cloning. Purified 16S rRNA PCR products were cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Cloning solution for each sample was prepared: 1µl $MgCl_2$ salt solution, 5.0µl purified PCR product and 0.8µl Topo vector. The solution was mixed carefully and incubated at room temperature for 25 minutes then 5µl of the solution was pipetted into tube containing TOPO 10 cells and incubated

on ice for 30 minutes. The TOPO 10 cells were shocked by placing them in 42°C water bath for 30 seconds and then immediately put on ice. A 170µl of SOC nutrient was added and the whole incubated at 37°C for 60 minutes on a stirring plate. Cells were placed on LB nutrient agar containing ampicillin and incubated at 37°C over night. Clones were picked randomly and each clone placed in separate wells in a 96-well plate containing LB broth and ampicillin. A clone library for each sample was constructed, prepared and incubated for 24 hours. Consequently, clones were amplified using the following PCR reaction mixture (15µl): dNTP (10mM) 0.20µl, 10x butter 1.50µl, M13 forward (100µM) 0.09µl, M13 reverse (100mM) 0.09µl, Teg polymerase (3U/µL) 0.12µl, Sterile dH_2O 13.0µl, clone culture from liquid LB broth 0.1µl. PCR amplification was performed using the following PCR protocol: 94°C for 2 min followed by 34 cycles at 94°C for 30 sec, annealing temperature ranging from 52°C for 50 sec each cycle and 72°C for 90 sec, with final extension period for 7 min at 72°C and with final temperature at 4°C. 5µl of PCR products were elecrophorized, for certification of clone insert size, on 1% agarose gel and visualized under UV light.

3.9 16S rRNA gene sequencing

Exo/SAP (Exonuclease I and Shrimp Alkaline Phosphatase) was used for the purification of 2.50µl PCR product prior to sequencing. This is essential in order to obtain a clean read, as the Exonuclease I will degrade any excess primer from the original PCR, while the SAP will de-phosphorylate any dNTP´s from the PCR. Around 50 clones were picked randomly from each clone library. Screening of the libraries was performed by sequencing with primer R.805 on ABI 3730 capillary sequence analyzer. This procedure was performed using the BigDye Terminator cycle sequencing kit (Applied Biosystems).

3.10 Data analysis of 16S rRNA sequencing

Data entry, editing, sequence alignment and analyzing were performed using sequencer v4.7 (Gene Codes Corporation, Ann Arbor, Mi, USA). Edited sequences were aligned using 98% minimum match. For the identification of closest relatives, sequences of the unrecognized inserts were compared to known 16S rRNA gene sequences using BLASTn search on the NCBI website through GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (52).

3.11 Simpson diversity index

Diversity was calculated using Simpson's diversity index. Both Simpson´s 1-D and 1/D are shown just to sharpen the differences in diversity between samples. For 1-D the diversity index gives a number between 0 and 1, the greater diversity nearer to 1. For 1/D the higher the number is the greater the diversity, but only up to the same number of clones analyzed. In general there is no difference between those calculations; n = the total number of organisms of a particular species and N = the total number of organisms of all species.

Equation 1. Calculation of Simpson´s diversity index

$$D = \frac{\sum n(n-1)}{N(N-1)}$$

3.12 Terminal restriction fragment length polymorphism (T-RFLP)

In this study the forward and reverse primers were both fluorescently-labeled with a different fluorescent dye. The "universal" bacterial selective primers F.9 and R.805 were used for amplification; the forward primer 9.F was fluorescent-labelled with a 5´-FAM terminal label and reverse primer 805.R was 5´-HEX-labelled. Primers used in this study produce around 800 base-pair fluorescent-labelled PCR products and the same primers as used for 16S rRNA gene analysis and clone sequencing.

PCR reaction for T-RFLP was performed using the following PCR program: 94°C for 5 minutes followed by 34 cycles of 94°C for 30 seconds, annealing temperature ranging from 45°C-55°C for 50 sec each cycle and 72°C for 90 sec, with final extension period for 10 minutes at 72°C. After amplification, PCR products were verified with electrophoresis in 1% agarose gel and visualized under UV light. The products were cut from the agarose and purified with GFX purification kit (Amersham Biosciences)

Labelled PCR products were digested with two tetrameric restriction enzymes (4 base-pair cutters) *Hae*III and *Alu*I (Fermentas, Hanover, MD, USA) in a 10µl reaction volume overnight 37°C. A 1µl of restriction enzyme (10U/µl, final conc. 1U), 1µl buffer, labeled 2µl PCR product and filled up to 10µl reaction volume with sterile H_2O. After digestion of the PCR products, restriction enzymes were heat-inactivated at 70°C for 30 minutes and then prepared for fragment analysis.

Digested PCR products were diluted in sterile dH_2O to 1:5, 1:10 and 1:20 for each analysis. Two micro liters of the dilutions were added to 8µl of GeneScan 500 LIZ internal size standard (Applied Biosystems, Warrington, UK) in formamide. The fragment analysis was carried out in ABI 3730 DNA capillary sequence analyzer (Applied Biosystems). Data analysis were carried out on the GeneMapper software (v4.0) (Applied Biosystems) using the AFLP analysis method. T-RF peaks below a threshold level of 40 fluorescent units were excluded except where a clear trend of same t-RF was detected in other samples.

Fragments generated between 35 to 500 base pairs were evaluated and counted in each sample. The relative area of each fragment peak in the profile was then calculated by dividing the respective peak area with the total peak area of all terminal restriction fragments in each sample. The T-RFLP profiles were also partially identified by comparison to sequences obtained from 16S rRNA gene sequencing analysis and to known reference sequences from Genebank (http://www.ncbi.nlm.nih.gov/Genbank/).

4. RESULTS

4.1 Culture studies

Cultures were performed to estimate the number of *Streptococcus mutans* and *Lactobacillus* sp. on selective agar media. These bacteria are known cariogenic bacteria and considered a good indicator bacteria for elevated risk of getting new caries (23). Viable counts of *S. mutans*, ranging from no growth in nine of the thirteen samples and $1.4x10^3$ cfu/ml in caries-free up to $9.2x10^5$ cfu/ml, in sample from early cavitated lesion (**Table 3**). Overall very limited bacterial growth was recovered from the caries-free samples and initial caries samples as well. Clear differences in the growth of *Lactobacillus* sp. were obtained, and only recovered from the four individuals that had obvious active caries, counts of *Lactobacillus* sp. ranged from $5.5x10^4$ cfu/ml to more than $1.0x10^5$ cfu/ml, $6.9x10^4$ cfu/ml on average.

Furthermore, total counts for bacteria were performed using blood agar plates, both under aerobic and anaerobic conditions. In samples from caries-free teeth no bacterial growth was obtained at all. In samples from initial caries teeth very limited growth was recovered and in two of five samples no bacterial growth was recovered at all. On average, anaerobic counts gave $6.5x10^5$ and total aerobic count was $9.3x10^5$ cfu/ml.

Table 3. Overview of the samples, clinical diagnosis and the bacterial counts of *S. mutans* and *Lactobacillus* sp.

Subject	Sample	Clinical diagnosis	Diagnodent reading	*S.mutans* (cfu/ml)	*Lactobacillus* (cfu/ml)
1	CF1	caries-free	11	ND	ND
2	CF2	caries-free	8	$1.4x10^3$	ND
3	CF3	caries-free	16	ND	ND
4	CF4	caries-free	10	ND	ND
5	CF5	caries-free	19	$1.6x10^3$	ND
6	CB1	initial caries	43	$6.2x10^3$	ND
7	CB2	initial caries	33	ND	ND
8	CB3	initial caries	24	ND	ND
9	CB4	initial caries	31	ND	ND
10	CA1	early cavitation	99	ND	$2.4x10^4$
11	CA2	early cavitation	99	ND	$9.8x10^4$
12	CA3	early cavitation	99	$4.4x10^4$	$5.5x10^4$
13	CA4	early cavitation	99	$9.2x10^5$	$1.0x10^6$

*ND, not detected in sample

Samples from deep dentine caries were also obtained but after repeated sampling and only negative results from cultures and PCR-based methods, this caries level was excluded from further analysis.

4.2 Oral bacteria detected with the 16S rRNA gene analysis method

Species identification and phylogenic analysis, alignment and comparison to known 16S rRNA sequences were performed, using the BLAST program search through the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). This application gave us identification of 114 taxa belonging to 5 different bacterial phyla. A total of 576 clones sequenced gave good and readable results from partially-sequenced clones using primer R.805. In total, 213 clones were analyzed from 5 caries-free samples, 178 clones from initial caries samples and 189 clones from early cavitated lesions. Members of 5 different phyla were obtained: Firmicutes, Actinobacteria, Fusobacteria, Bacteriodetes and Proteobacteria. The distribution of clones by phylum varied between individual samples but on average the 16S rRNA results showed that members of Firmicutes bacteria were predominant in all sample types. The proportion of Firmicutes bacteria ranged from 50% in caries free, increasing to 76% in initial caries and was 81% of the total flora in cavitated lesions. Only species of the phylum Actinobacteria were also found in all types of samples, 18% of the bacteria in initial caries, 19% in cavitated lesions and 29% in caries-free samples. Proteobacteria were 10% of all clones in samples from caries-free sites, 5% of clones in initial caries but not found in early cavitated lesions. Members of the phylum Fusobacteria were found in caries-free and initial-caries sites and one clone of *Prevotella tannerae* belonging to Bacteriodetes was also found in one caries free sample. In cavitated samples the microflora was only composed of Firmicutes bacteria, 81%, and remaining 19% were Actinobacteria. Tables 4-6 show the composition of each environment, caries free surface, initial caries and cavitated caries. Considerable variation in microflora composition was obtained in different environment.

4.2.1 Dominant bacteria on caries free surface

In total a 213 clones were sequenced from the five samples examined from the surfaces of caries-free teeth. Table 4 shows the composition of the microflora in caries free sites and the number of individual phylotypes identified. In total 63 phylotypes were detected in these samples. Overall, *Streptococcus* sp. was generally predominant in these caries-free samples, 33% of all clones,

with individual variation as expected. This environment was dominated by *S. mitis*, *S. mutans*, *S. gordonii*, and *S. sanguinis.* Other phylotypes were also detected in multiple copies such as 25 clones of *Rothia dentocariosa*, 12 clones of *Veilonella parvula.* Eleven clones of *Actinomyces naeslundi* and several clones of other *Actinomyces* sp. as well and 8 clones of *Haemophilus parainfluenze*, 6 clones of *Fusobacterium nucleatum subsp. nucleatum* and 5 clones of *Micrococcus luteus.*

4.2.2 Dominant bacteria in samples from initial caries surface

Table 5 shows the phylotypes detected in the samples taken from initial caries sites. Totally 33 phylotypes were identified 178 clones analyzed. The majority of phylotypes identified were related to only two phyla, Firmicutes bacteria and Actinobacteria. As in caries-free samples, the majority of clones identified were related to *Streptococcus* sp. Most abundant were clones related to *S. mitis*, or 41 clones. Other Streptococci were also frequently found. The most obvious changes in bacterial composition from caries-free to initial caries are probably the number of *Lactobacillus* sp. detected in initial caries samples but not one clone of *Lactobacillus* sp. was detected in caries-free sample. Other species such as *Rothia mucilaginosa* and *R. dentocariosa* were also found in multiple copies as were several clones of *Actinomyces* sp.

4.2.3 Dominant bacteria in samples from early cavitated lesions

In the four samples from the early cavitated lesions, 36 phylotypes were detected from 189 successfully analyzed clones. The majority of clones identified were related to various *Lactobacillus* sp. particularly in two of four samples, indicating that the environment was changing to being more acidic and thus more attractive to acid-tolerant bacteria. In total 27 clones were identified as *Olsenella profusa*, and only found in one particular sample. Other phylotypes also only detected in that particular environment, such as *Scardovia inopinata* and *Parascardovia denticolens*. Phylotypes related to *Streptococcus* sp. were not as frequently detected as in caries-free and initial caries samples. However, in sample CA3 around 50% of sequenced clones were related to *S. mutans* but some individual variation was observed. Additionally, there were several uncultivable and unidentified bacteria detected in most samples.

Table 4. Bacteria identified in on caries free surface by 16S rRNA analysis, showing the similarity (%) to its closest relative in the NCBI database and the number of clones analyzed.

#	Acccess number	Closest database match	Similarity (%)	No. of clones						Phylum
				Subjects						
				Total	CF 1	CF 2	CF 3	CF 4	CF 5	
1	AY207066.1	*Actinobaculum* sp. *P2P_19 P1*	99	2		2				Actinobacteria
2	AF479270.1	*Actinomyces israelii*	95	1			1			Actinobacteria
3	AJ234048.1	*Actinomyces naeslundii*	98	16	1	1	14			Actinobacteria
4	EF474012.1	*Actinomyces* sp. *C162*	99	2			1	1		Actinobacteria
5	EF474012.1	*Actinomyces* sp. *C162 16S*	99	1			1			Actinobacteria
6	AY008315.1	*Actinomyces* sp. *oral clone EP011*	99	1	1					Actinobacteria
7	AY349363.1	*Actinomyces* sp. *oral clone IO076*	99	1		1				Actinobacteria
8	AY349365.1	*Actinomyces* sp. *oral clone IP073*	99	2			2			Actinobacteria
9	X82453.1	*Actinomyces viscosus*	98	1			1			Actinobacteria
10	AF543288.1	*Corynebacterium matruchotii sequence*	99	1		1				Actinobacteria
11	DQ659431.1	*Micrococcus luteus*	99	5		1		4		Actinobacteria
12	EF187229.1	*Micrococcus luteus strain AUH1*	97	2				2		Actinobacteria
13	EU135689.1	*Nesterenkonia* sp. *YIM C732*	100	1				1		Actinobacteria
14	AJ717364.1	*Rothia dentocariosa*	99	25		5	19		1	Actinobacteria
15	DQ409140.1	*Rothia mucilaginosa*	99	1					1	Actinobacteria
16	AF183402.1	*Prevotella tannerae*	99	1				1		Bacteroidetes
17	AY879308.1	*Abiotrophia defectiva*	99	1		1				Firmicutes
18	EU143680.1	*Bacillus alkalidiazotrophicus*	97	1				1		Firmicutes
19	AB294141.1	*Clostridium thiosulfatireducens*	99	1				1		Firmicutes
20	DQ333294.1	*Enterococcus casseliflavus*	100	1				1		Firmicutes
21	AY349376.1	*Eubacterium* sp. *oral clone IR009*	99	4		4				Firmicutes
22	AF287774.1	*Firmicutes* sp. *oral clone CK051*	99	1		1				Firmicutes
23	Y13364.1	*Gemella sanguinis*	99	2					2	Firmicutes
24	AY879305.1	*Granulicatella adiacens*	99	1		1				Firmicutes
25	X87152.1	*Johnsonella ignava*	98	1		1				Firmicutes
26	EF120374.1	*Lactobacillus crispatus*	99	1				1		Firmicutes
27	DQ085279.1	*Paenibacillus ruminocola*	94	1				1		Firmicutes
28	AY878646.1	*Selenomonas noxia*	98	2		2				Firmicutes
29	EU071614.1	*Staphylococcus epidermidis*	99	1		1				Firmicutes
30	AY584476.1	*Streptococcus cristatus*	99	3		3				Firmicutes
31	AJ514237.1	*Streptococcus gordonii*	99	8	7	1				Firmicutes
32	AJ295853.1	*Streptococcus mitis*	100	29			2	4	23	Firmicutes
33	DQ677784.1	*S. mutans*	100	11	5	6				Firmicutes
34	AY188352.1	*Streptococcus salivarius*	100	3					3	Firmicutes
35	AY691542.1	*Streptococcus sanguinis*	100	12	4		4	1	3	Firmicutes
36	AY134908.1	*Streptococcus* sp. *oral strain 12F*	100	2	1				1	Firmicutes
37	AY607176.1	*Uncultured Clostridia bacterium*	98	1				1		Firmicutes
38	EF703035.1	*Uncultured Clostridium* sp.	100	1				1		Firmicutes
39	EU112139.1	*Uncultured Streptococcus* sp.	97	1			1			Firmicutes
40	AY806535.1	*Uncultured Streptococcus* sp.	96	1					1	Firmicutes
41	AY807108.1	*Uncultured Streptococcus* sp.	97	1					1	Firmicutes
42	DQ188777.1	*Uncultured Veillonella* sp.	95	1		1				Firmicutes
43	DQ188777.1	*Uncultured Veillonella* sp.	97	1					1	Firmicutes
44	DQ188779.1	*Uncultured Veillonella* sp.	96	1					1	Firmicutes
45	AY995769.1	*Veillonella parvula*	99	12	2	3			7	Firmicutes
46	AJ006962.1	*Fusobacterium alocis*	99	2				2		Fusobacteria
47	AJ810281.1	*Fusobacterium nucleatum* subsp.*polymorphum*	99	6	5	1				Fusobacteria
48	AF287813.1	*Leptotrichia* sp. *oral strain FAC5*	99	1		1				Fusobacteria
49	AY807024.1	*Uncultured Leptotrichia* sp. *clone IS024B37*	99	4	1			2	1	Fusobacteria
50	AM293676.1	*Acinetobacter lwoffii*	100	1		1				Proteobacteria
51	AF320620.1	*Eikenella corrodens*	99	1	1					Proteobacteria
52	EU083530.1	*Haemophilus parainfluenzae*	99	8				1	7	Proteobacteria
53	AY365450.1	*Haemophilus parainfluenzae*	92	1					1	Proteobacteria
54	EU057873.1	*Janthinobacterium* sp. *15K*	99	1				1		Proteobacteria
55	AJ582227.1	*Loktanella vestfoldensis*	98	1				1		Proteobacteria
56	EF194104.1	*Proteus mirabilis*	99	1				1		Proteobacteria
57	EU034528.1	*Pseudomonas* sp. *LFX-15B1*	99	3		1		2		Proteobacteria
58	AF538712.1	*Roseomonas mucosa*	99	1				1		Proteobacteria
59	DQ016725.1	*Uncultured Lautropia* sp. *clone 2.15*	100	3		2			1	Proteobacteria
60	DQ016725.1	*Uncultured Lautropia* sp. *clone 2.15 16S*	89	1					1	Proteobacteria
61	DQ016677.2	*Uncultured bacterium clone 7.69*	98	2	1	1				Unidentif. eubacterium
62	AY375144.1	*Uncultured bacterium clone D5*	99	3				3		Unidentif. eubacterium
63	DQ129951.1	*Uncultured bacterium clone LCKS40-B20*	99	4				4		Unidentif. eubacterium
				213	29	43	46	39	56	

Table 5. Bacteria identified on initial caries surfaces by 16S rRNA analysis, showing the similarity (%) to its closest relative in the NCBI database and the number of clones analyzed.

#	Access number	Closest database match	Similarity	No. Of clones					Phylum
					Subject				
			(%)	Total	CB1	CB2	CB3	CB4	
1	AJ234052.1	*Actinomyces naeslundii*	99	1	1				Actinobacteria
2	X81063.1	*Actinomyces* sp.	99	2	1	1			Actinobacteria
3	AY349363.1	*Actinomyces* sp. *oral clone IO076*	99	5	5				Actinobacteria
4	AJ234056.1	*Actinomyces viscosus*	98	1	1				Actinobacteria
5	AJ717364.1	*Rothia dentocariosa 16S rRNA*	100	7	3	1		3	Actinobacteria
6	DQ409140.1	*Rothia mucilaginosa*	99	15			15		Actinobacteria
7	AB271749.1	*Bacillus smithii*	99	1				1	Firmicutes
8	L14326.1	*Gemella haemolysans*	99	1				1	Firmicutes
9	AY005051.1	*Gemella* sp. *oral strain C24KA*	99	1			1		Firmicutes
10	AY879304.1	*Granulicatella adiacens*	100	8	5	2	1		Firmicutes
11	EF460495.1	*Lactobacillus gasseri*	100	4		4			Firmicutes
12	AY283269.1	*Lactobacillus iners clone FX9-5*	99	25		25			Firmicutes
13	DQ256277.1	*Lactobacillus reuteri isolate LP970*	96	1				1	Firmicutes
14	AB158767.1	*Lactobacillus vaginalis*	99	2				2	Firmicutes
15	AY281088.1	*Streptococcus gordonii*	99	7	5	2			Firmicutes
16	AY485603.1	*Streptococcus infantis*	99	3	1		2		Firmicutes
17	DQ232531.1	*Streptococcus intermedius*	100	1		1			Firmicutes
18	AY518677.1	*Streptococcus mitis*	100	41	8	2	3	28	Firmicutes
19	DQ677788.1	*S. mutans strain ChDC YM15*	100	4	2		1	1	Firmicutes
20	CP000410.1	*Streptococcus pneumonia*	97	1				1	Firmicutes
21	AY188352.1	*Streptococcus salivarius*	99	6	1	3	1	1	Firmicutes
22	CP000387.1	*Streptococcus sanguinis*	99	2	1			1	Firmicutes
23	AY995245.1	*Uncultured Granulicatella* sp. *clone FX22W27*	100	1				1	Firmicutes
24	DQ016843.2	*Uncultured Streptococcus* sp. *clone 8.8*	98	19			18	1	Firmicutes
25	AY807114.1	*Uncultured Veillonella* sp. *clone IS027B18*	99	1			1		Firmicutes
26	AF287782.1	*Veillonella* sp. *oral clone AA050*	99	7	5	1		1	Firmicutes
27	DQ440557.1	*Fusobacterium nucleatum strain Ulm 8*	99	1		1			Fusobacteria
28	AY008309.1	*Leptotrichia* sp. *oral clone BU064*	99	1		1			Fusobacteria
29	AB291890.1	*Sphingomonas* sp. *Pd-S-(l)-m-D-3(6)*	100	1		1			α-Proteobacteria
30	AJ290755.2	*Haemophilus pittmaniae*	99	1				1	γ-Proteobacteria
31	AM411997.1	*Pseudomonas* sp.	100	5		1		4	γ-Proteobacteria
32	EU009183.1	*Shigella dysenteriae*	100	1	1				γ-Proteobacteria
33	AY807157.1	*Uncultured Haemophilus* sp. *clone IS028B87*	99	1			1		γ-Proteobacteria
				178	40	46	44	48	

Table 6. Bacteria identified in early cavitated lesions by 16S rRNA analysis, showing the similarity (%) to its closest relative in the NCBI database and the number of clones analyzed.

#	Access Number	Closest database match	Similarity (%)	No. of clones					Phylum
				Subject					
				Total	CA1	CA2	CA3	CA4	
1	AF292374.2	*Olsenella profuse*	100	27		27			Actinobacteria
2	D89332.1	*Scardovia inopinata*	99	3			3		Actinobacteria
3	D89331.1	*Parascardovia denticolens*	99	3		3			Actinobacteria
4	D89332.1	*Scardovia inopinata*	90	1			1		Actinobacteria
5	D89332.1	*Scardovia inopinata*	95	1			1		Actinobacteria
6	AY594189.1	*Rothia dentocariosa*	99	1			1		Actinobacteria
7	EF460495.1	*Lactobacillus gasseri*	100	39	17			22	Firmicutes
8	DQ677788.1	*S. mutans*	100	25			25		Firmicutes
9	DQ346420.1	*Uncultured Veillonella*	99	14		2	12		Firmicutes
10	AB288235.1	*Lactobacillus rhamnosus*	99	14	7			7	Firmicutes
11	EF460497.1	*Lactobacillus vaginalis*	99	13	13				Firmicutes
12	AB362758.1	*Lactobacillus pentosus*	100	11				11	Firmicutes
13	AJ250074.1	*Lactobacillus* sp.	99	7			1	6	Firmicutes
14	AF287793.1	*Selenomonas sputigena*	99	3		3			Firmicutes
15	AF439643.1	*Veillonella* sp.	98	2			2		Firmicutes
16	AY807653.1	*Uncultured Veillonella* sp.	93	2			2		Firmicutes
17	AY349383.1	*Lactobacillus* sp.	99	2		2			Firmicutes
18	AB262735.1	*Lactobacillus parafarraginis*	99	2	2				Firmicutes
19	EF533990.1	*Lactobacillus gasseri*	98	2	1			1	Firmicutes
20	AY995769.1	*Veillonella parvula*	97	1			1		Firmicutes
21	DQ188776.1	*Uncultured Veillonella* sp.	95	1			1		Firmicutes
22	DQ857092.1	*Uncultured Lactobacillus* sp.	94	1				1	Firmicutes
23	DQ857092.1	*Uncultured Lactobacillus* sp.	96	1	1				Firmicutes
24	AY923125.1	*Streptococcus* sp. *oral clone*	96	1			1		Firmicutes
25	DQ677720.1	*S. mutans*	93	1		1			Firmicutes
26	AB294730.1	*S. mutans*	96	1			1		Firmicutes
27	DQ677734.1	*S. mutans*	90	1			1		Firmicutes
28	AF287793.1	*Selenomonas sputigena*	97	1		1			Firmicutes
29	AY349404.1	*Selenomonas* sp. *oral clone*	97	1			1		Firmicutes
30	AY349403.1	*Selenomonas* sp. *oral clone*	97	1		1			Firmicutes
31	AF287795.1	*Selenomonas* sp. *oral clone*	99	1		1			Firmicutes
32	AF243163.1	*Lactobacillus* sp.	97	1				1	Firmicutes
33	AY590777.1	*Lactobacillus plantarum*	97	1				1	Firmicutes
34	Y19168.1	*Lactobacillus perolens*	99	1	1				Firmicutes
35	AB295648.1	*Lactobacillus johnsonii*	95	1				1	Firmicutes
36	AF385570.1	*Eubacterium* sp. *oral clone EH006*	99	1		1			Firmicutes
				189	42	42	54	51	

4.3 Bacterial diversity

4.3.1 Simpson´s diversity index

The number of phylotypes identified, varied considerably between individual samples as expected, from 10 to 25 phylotypes at the most. Generally individual samples were composed by few predominated species. Overall, 1-D diversity ranged from 0.569-0.945 in individual samples, lowest in sample from cavitated lesion CA2 and highest in sample from caries-free surface CF4. On average, the diversity was greatest in caries-free samples ranging from 0.723-0.945 with an average value of 1-D = 0.86. The diversity decreased in initial with caries the average value of 1-D was 0.739 and in cavitated carious lesions the diversity was 0.686 (**Table 7**).

4.3.2 Coverage of samples

Coverage was calculated for species detected in the samples to evaluate the proportion of species detected of the potential number of species if all clones were sequenced. Overall, a lower coverage was obtained in individual samples with greater diversity. The average coverage ranged from 72.0% in caries-free, 81.6 % in initial-caries to 87.1% in cavitated caries.

Table 7. Clones analyzed in each sample and bacterial diversity calculated using Simpson´s diversity index (D) and the coverage of identified species.

		Caries free						Initial caries					Cavitated caries				
		CF1	CF2	CF3	CF4	CF5	**Average**	CB1	CB2	CB3	CB4	**Average**	CA1	CA2	CA3	CA4	**Average**
Nr. of clones analyzed		29	43	46	39	56	**42.6**	40	46	44	48	**44.5**	42	42	51	54	**47.25**
Nr. of Phylotypes observed		10	25	10	25	20	**18**	15	14	10	16	**13.75**	8	10	16	9	**10.75**
Nr. of singletons		6	18	5	17	13	**11.8**	7	8	6	12	**8.25**	4	5	11	5	**6.25**
Good´s Coverage (%)		79.3	58.1	89.1	56.4	76.8	**72.0**	82.5	82.6	86.4	75	**81.6**	90.5	88.1	79.6	90.2	**87.1**
Simpson´s diversity index	1-D	0.851	0.935	0.723	0.945	0.833	**0.86**	0.900	0.683	0.707	0.666	**0.739**	0.715	0.569	0.728	0.733	**0.686**
	1/D	6.73	15.28	3.61	18.33	5.98	**9.99**	10.00	3.158	3.408	2.992	**4.890**	3.514	2.321	3.682	3.742	**3.315**
***Streptococcus mutans* (+)/(-)**	Culture	(-)	(+)	(-)	(+)	(+)	3/5	(+)	(-)	(-)	(-)	1/4	(+)	(+)	(+)	(+)	4/4
	16S rRNA	(+)	(+)	(-)	(-)	(-)	2/5	(+)	(-)	(+)	(+)	3/4	(-)	(+)	(+)	(-)	2/4
***Lactobacillus* sp. (+)/(-)**	Culture	(-)	(-)	(-)	(-)	(-)	0/5	(-)	(-)	(-)	(-)	0/4	(+)	(+)	(+)	(+)	4/4
Identified/not identified	16S rRNA	(-)	(-)	(-)	(+)	(-)	1/5	(-)	(+)/29	(-)	(+)	2/4	(+)	(+)	(+)	(+)	4/4

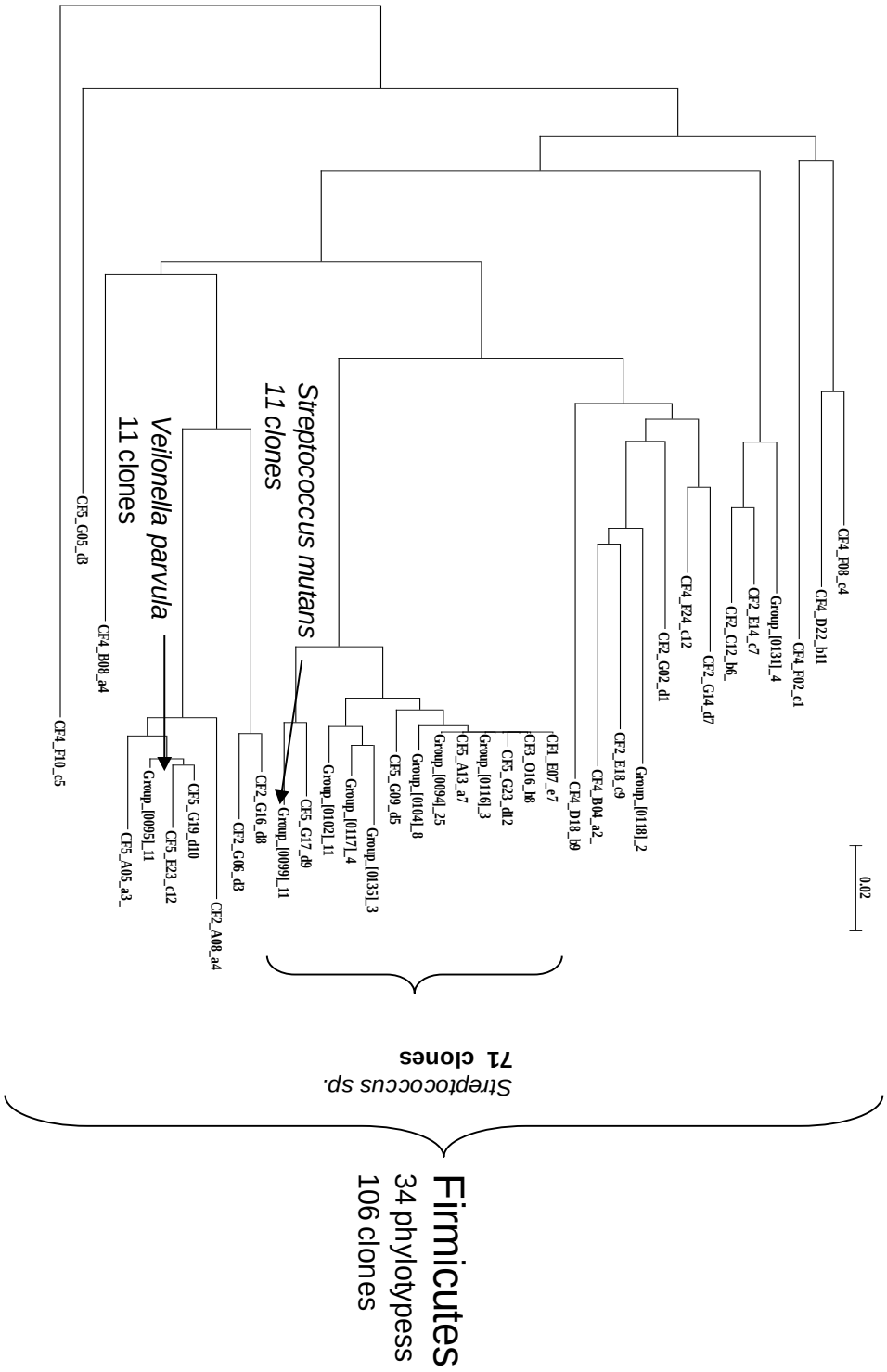

Figure 4. Phylogenic tree of Firmicutes bacteria from caries-free sites. Continued on next page

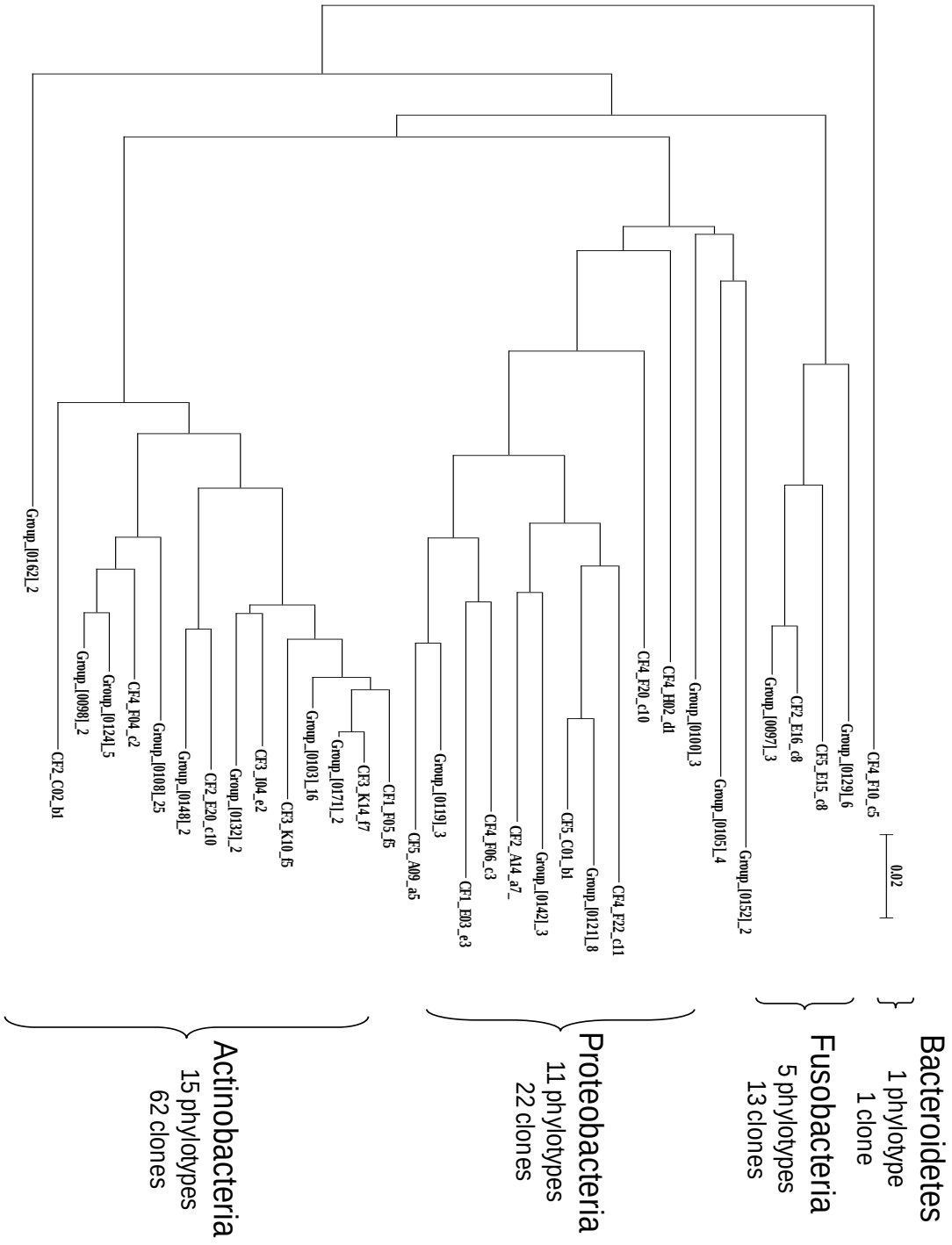

Figure 5. Continued from page 45. Phylogenic tree of bacteria, other than Firmicutes, from caries-free sites.

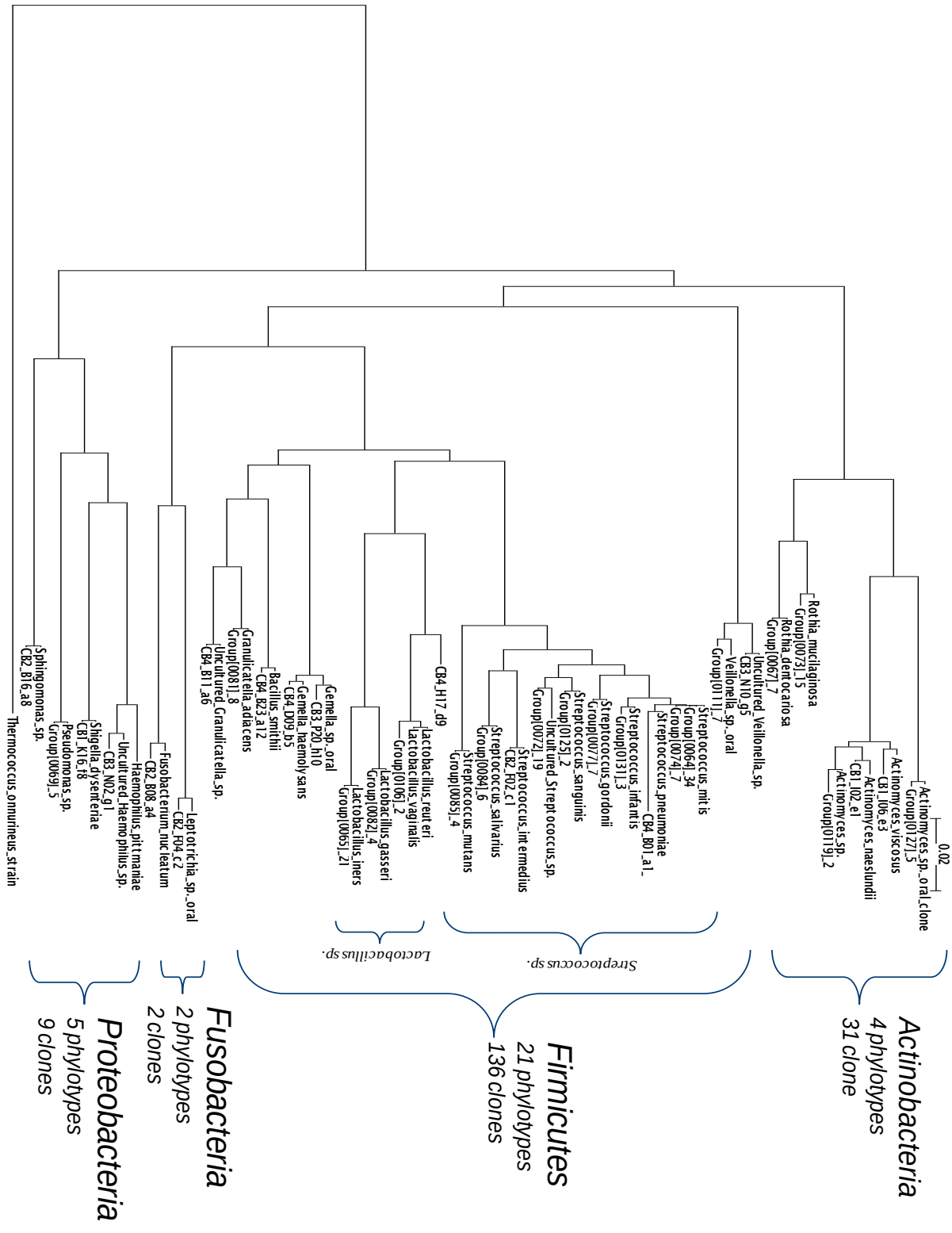

Figure 6. Phylogenic tree of bacteria from initial caries samples

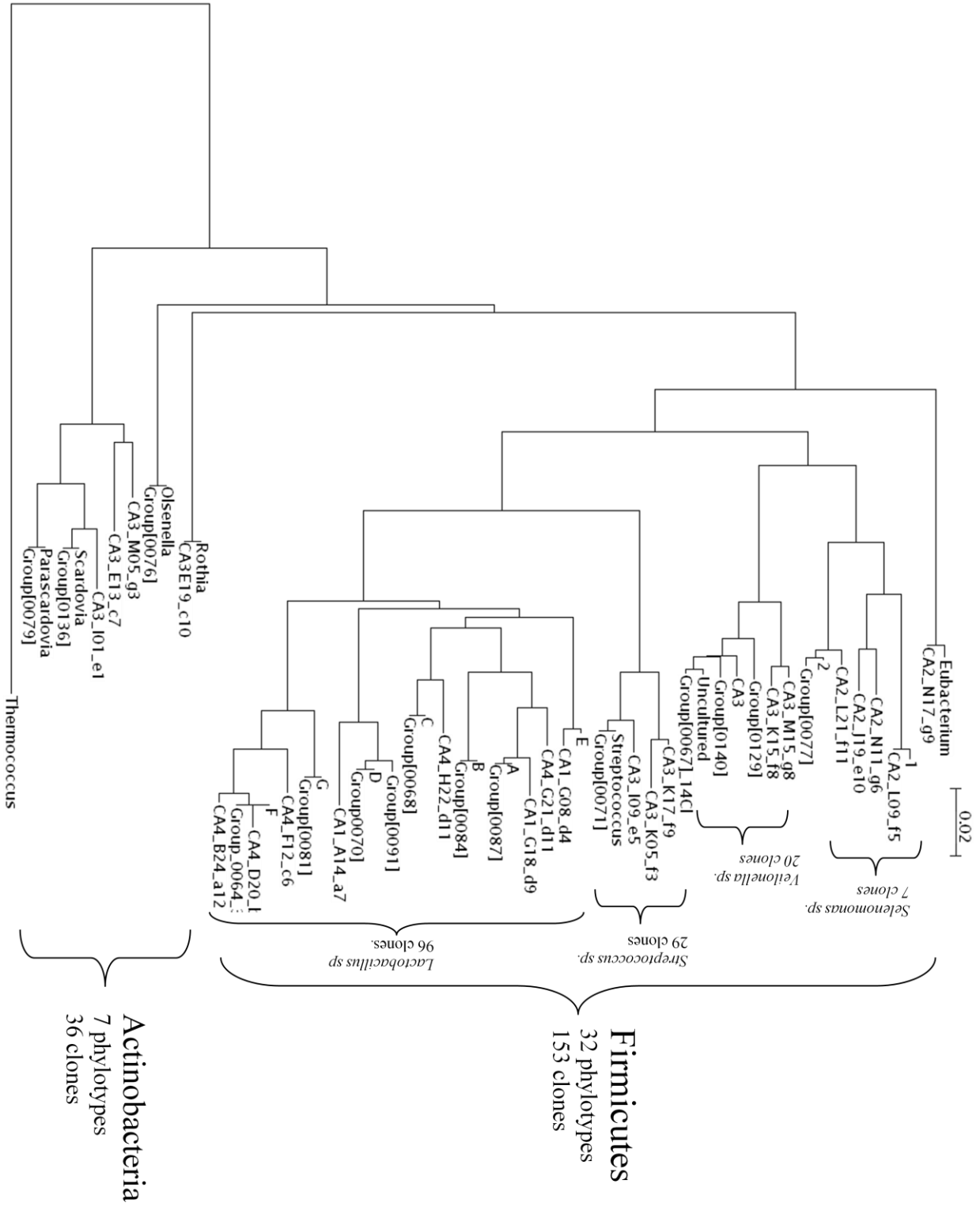

Figure 7. Phylogenic tree of bacteria from early cavitated lesions.

4.4 Terminal restriction fragment length polymorphism (T-RFLP)

Results from fragments produced by FAM labeled F.9 and *HaeIII* were the T-RFLP profiles that were consistent and gave reliable results for most of the samples analyzed. Representative electropherograms for three samples are shown in fig. 9.A - C but at least 3 repeated profiles were generated for each sample. From these T-RFLP profiles of 12 samples analyzed, four from caries-free (CF) tooth surface, four samples from initial caries (CB) and four from early cavitated caries (CA) terminal restriction fragments were counted and relative abundance of each fragment calculated from the fluorescent signal. Column graphs were made from these electropherograms, showing the size of terminal fragments in base pairs and the relative proportion of peak area. The proportional peak area for each fragment in each sample indicates partially the quantity of related species or group of species in samples. There was a considerable variation in the T-RFLP profiles obtained of the 12 samples analyzed, four from caries-free tooth surface, four samples from initial caries and four from early cavitated lesions. Fig. 10-12 show the relative peak area for each sample. These column graphs show that, in most samples, large proportion of the calculated peak area often belong to one or two T-RFs and variable number of fragments with less peak area.

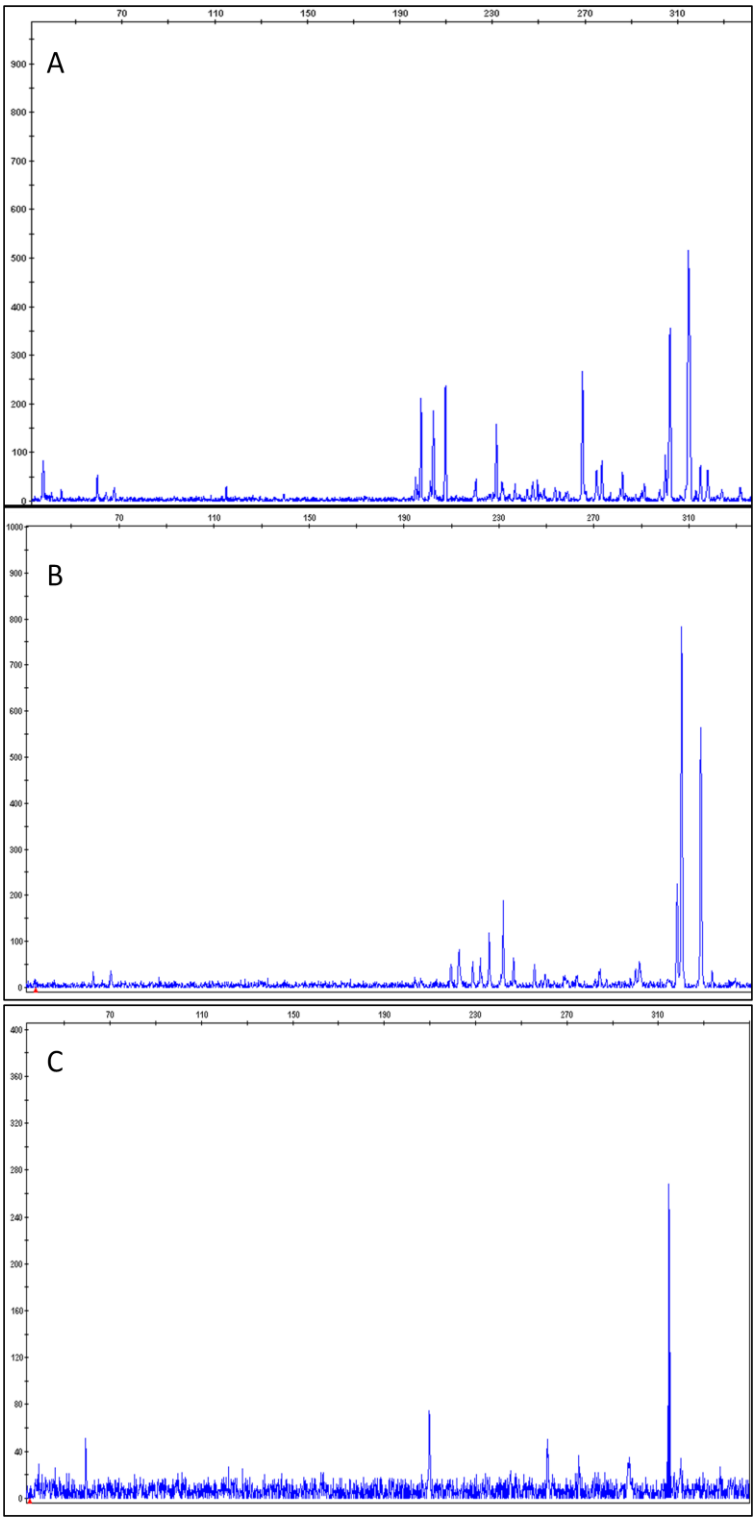

Figure 8. A-C Representative electrophereograms of T-RFLP patterns for three samples from: **A**. Caries-free surface(CF1) **B**. Initial caries (CB1) and **C** early cavitated lesion. This pattern was produced by primer FAM-F.9 and restriction enzyme *HaeIII*. Y-axis show the fluorescent signal and X- axis the fragment size in base pairs.

The proportional peak area for each fragment in each sample indicates, partially, the quantity of related species or group of species in the samples. In most samples one or two terminal fragments were obtained in large quantity and there were a variable number of fragments with less peak area. Considerable variation was obtained in fragment number, both between type of samples and as well between individual samples (Table 8). The lowest number of T-RFs was obtained in sample from early cavitated lesions and the highest number of T-RF´s in samples from healthy tooth surface. The number of T-RFs can be viewed as an indication of the bacterial diversity in each sample. Thus, indicating that the bacterial diversity is much greater in samples from caries free surface than from cavitated caries lesions. Furthermore, on average the greatest number of fragments was obtained in samples from caries-free surface (Table 8). A total of 20 distinctive restriction fragments were detected in sample caries-free samples, 25 fragments from initial caries and 18 from cavitated caries.

Terminal fragment size in the 12 samples was between 36 base pairs (bp) up to 487 base pairs but T-RFs of the same fragment size were repeatedly observed and commonly found in many samples. Terminal fragments of 232 bp. size and 307 bp size were most frequently detected. A 305bp size fragment was also frequently detected.

Of the 20 different terminal restriction fragments detected in caries-free samples, five were commonly detected, 199bp, 210bp, 232bp, 307bp and 315 base pairs, in all four samples. Only three T-RFs were commonly found in all four samples of initial caries sites, fragments 232bp, 305bp and 307bp. In samples from cavitated lesion, fragments detected were more distinctive for individual samples, only the 63bp, 329bp and 343bp size fragment were detected in more than once. The remaining fragments were only detected once.

Results for ROX-labeled R.805 primer were not as expected. Background “noise” was constantly high and making it difficult to distinguish the background from real peaks in most cases. Potential peaks, with signal within 100 fluorescent units, from the background. This problem was continuous throughout all repeated runs and made comparison of ROX-labeled R.805 profiles and FAM-labeled 9.F forward impossible. Therefore, T-RFLP results for the R.805 primer were excluded and only the data from the forward primer 9.F was validated. The T-RFLP results for the R.805 primer were consequently excluded.

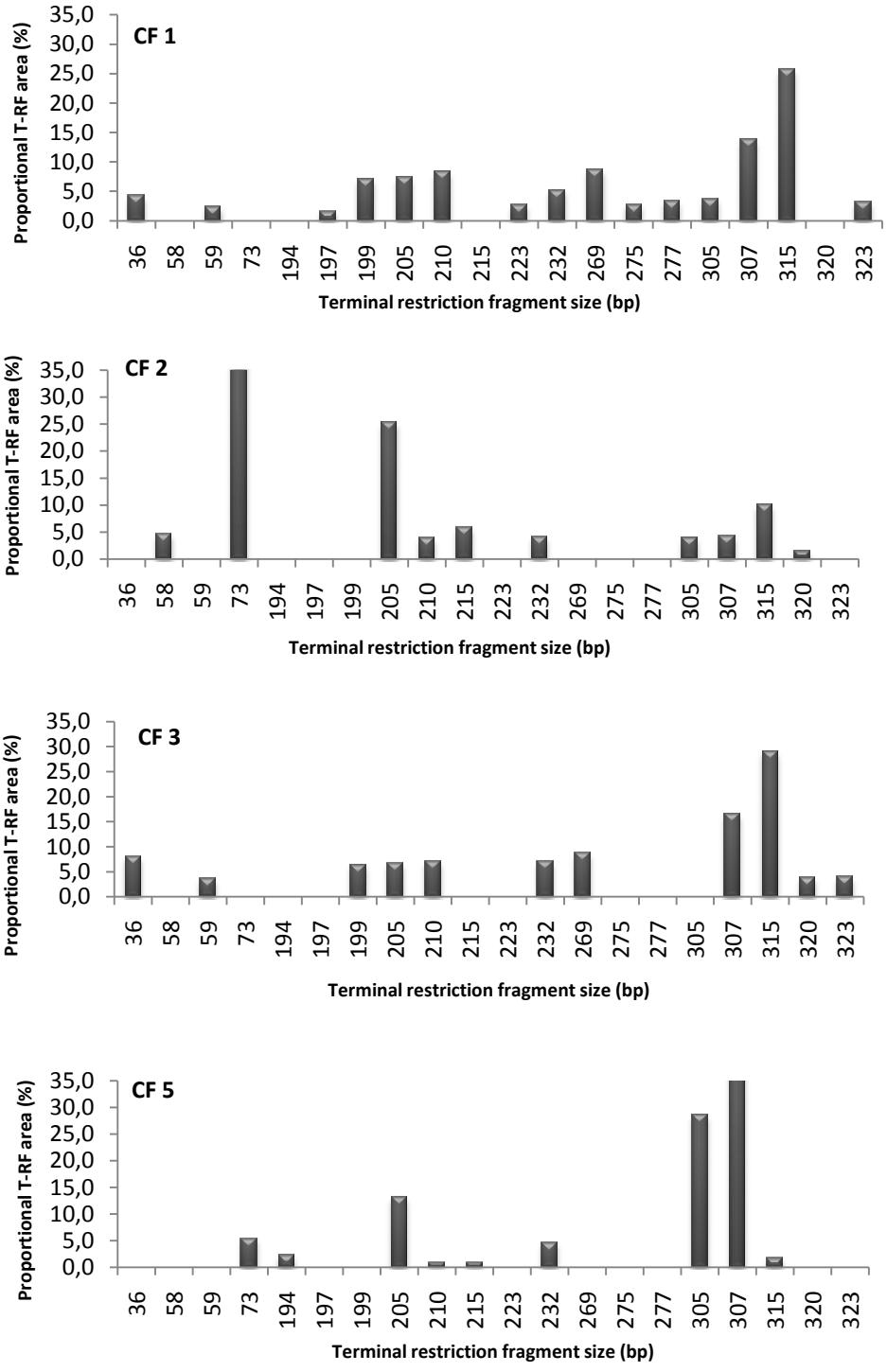

Figure 9. The size and relative abundance of terminal restriction fragments pattern from healthy sites.

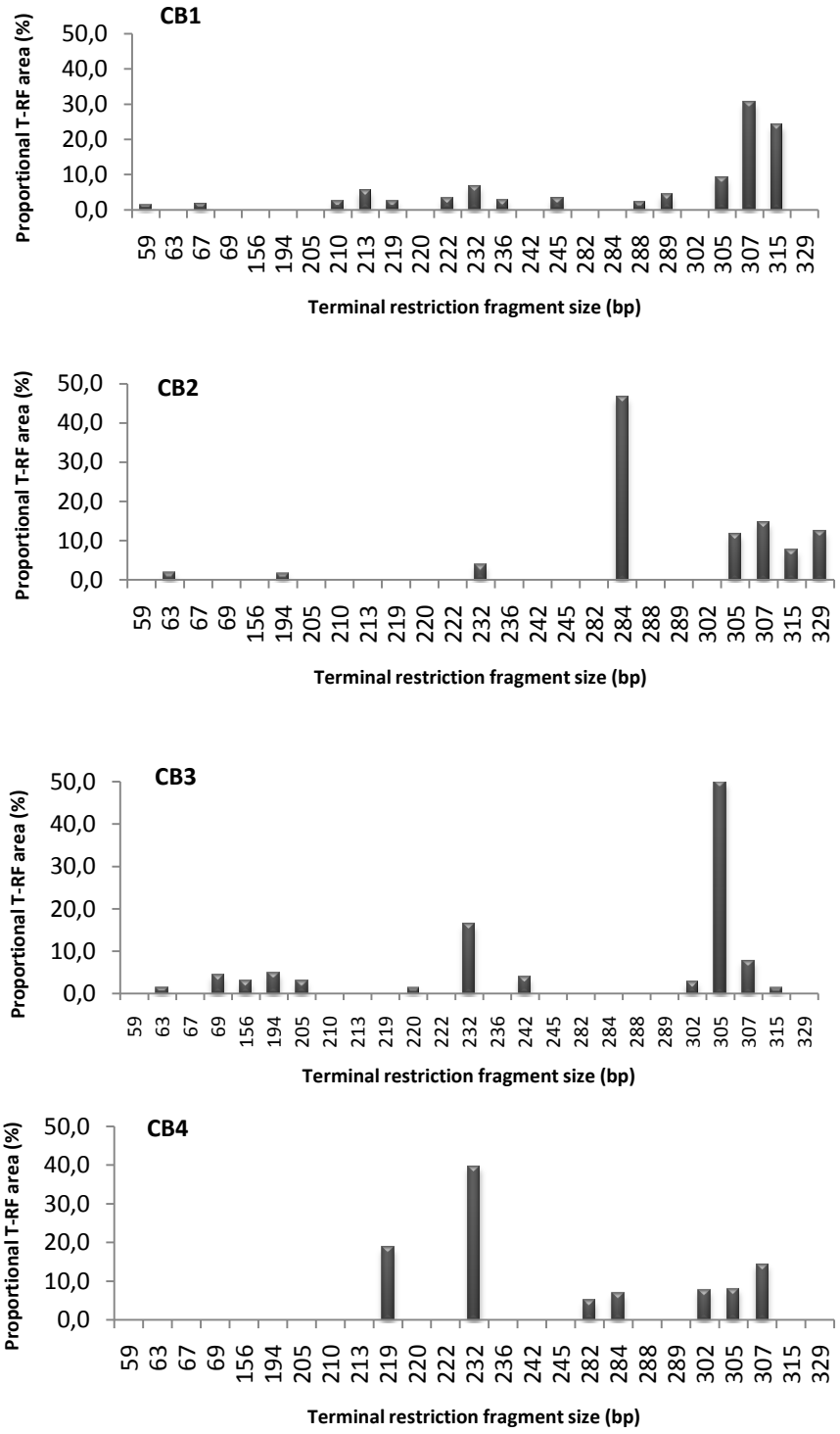

Figure 10. The size and relative abundance of terminal restriction fragments from initial caries sites.

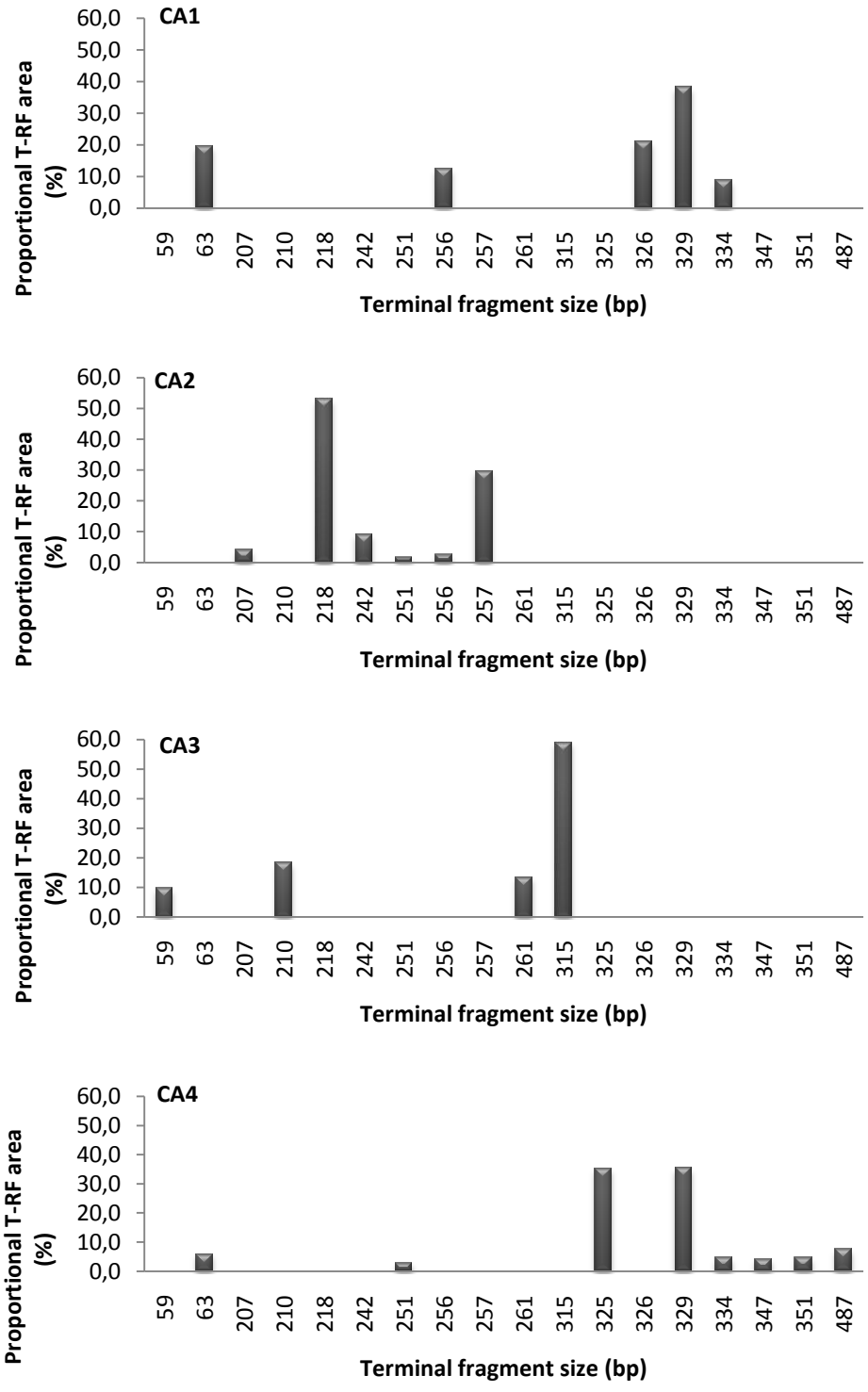

Figure 11. The size and relative abundance of terminal restriction fragments from early cavitated sites.

Table 8. The number of terminal restriction fragments (T-RFs) in each sample and sample type.

	#	Sample	Number of T-RFs			
			T-RFs/sample	Unique T-RFs	Average	Total in all samples
Caries free	1	CF1	15	20	11.25	
	2	CF2	10			
	3	CF3	11			
	4	CF5	9			
initial caries	5	CB1	14	25	9.75	46
	6	CB2	6			
	7	CB3	8			
	8	CB4	11			
cavitated caries	9	CA1	5	18	5.25	
	10	CA2	6			
	11	CA3	4			
	12	CA4	8			

In samples from healthy sites (CF) the 232 bp size could belong to *Rothia mucilaginosa* or *R. denticariosa*, 305 and 307bp fragment to various species of streptococci such as *S. mitis, S. cristatus, S. infantis* or *S. oralis.*, the 73 bp fragment matches to *Actinomyces naeslundii* and the 205 bp could belong to *Haemophilus parainfluenzae.*

Terminal fragment patterns in initial caries (CB) were not much different from those found in healthy sites. On average 9.75 T-RFs per sample and fragments 305, 307 and 232bp were all detected in relatively high proportions. In CB2 the 284 bp. fragment could belong to *Lactobacillus iners*. The 315 bp fragment detected in CB1 and CB2, can be matched to *S.mutans* and *S. gordonii* and the 329 bp. size fragment identified in CB2 could belong to *Lactobacillus* sp.

In samples from early cavitated lesions (CA) relatively few terminal fragments were obtained in each sample, on average 5.25 fragments per sample. In these samples one or two fragments were over 50% of the total fluorescent signal. The 315bp size fragment probably belongs to *S. mutans*. Terminal fragments of sizes 63 bp., 242bp, 325bp, 329bp and 334bp, could all match various *Lactobacillus* species and the 218 bp. fragment in sample CA2 matched the

sequence of *Olsenella profuse.* Other fragments, less abundant, such as 256bp and 257bp could match *Scardovia inopinata* and *Parascardovia denticoalens* and 210bp to *Veillonella* sp. or *Selenomonas* sp.

In total, 46 different fragment sizes were detected in all 12 samples, indicating the high bacterial diversity in dental plaque samples. This, however, does not give the complete picture of the diversity of these samples. Group of related species often have the same restriction site for the same restriction enzyme in the 16S rRNA gene and thus the size of the terminal fragment detected can be exactly the same for two separate species and 16S rRNA gene analysis gives the better picture.

5. DISCUSSION

5.1 General discussion

Investigating and diagnosing the causative organisms of infectious diseases has traditionally focused on one, or a small number, of suspected pathogens. Even when samples are taken from sites where a complex mixture of species coexists, the diagnostic methods aim at finding a specific pathogen. This is usually an exogenous pathogen that has either gained entry to the body or transferred to a new site, where it causes disease, from another site in the body where it may be commensal. Remaining organisms in a clinical sample, used for diagnosing an infection, are considered part of the "normal flora", common residents of the sampling site. Dental caries is, on the other hand, an unusual type of infectious disease, being a truly endogenous infection. The bacteria that have generally been associated with caries belong to the normal flora of the oral cavity, making identification of specific pathogens more complicated. Endogenous infections may occur when members of the resident flora obtain a selective advantage over other species disturbing the homeostatic balance of the biofilm with consequent succession towards a more pathogenic bacteria composition. Relatively easy access to the oral cavity has prompted detailed research into the association of biofilm composition and disease, most particularly in the field of periodontology (15, 59, 60, 70).

In general, the dental plaque covering tooth enamel supports many micro-ecosystems of diverse groups of bacteria. In a biofilm such as dental plaque, microorganisms are in close proximity with one another and interact as a consequence. These interactions can be beneficial to one or more of interacting populations, while others can be antagonistic. Microbial metabolism within the plaque will produce localized gradients in factors affecting the growth of other species, ranging from depletion of essential nutrients with the simultaneous accumulation of

toxic or inhibitory by-products, to the consumption of oxygen enabling the growth of obligate anaerobes. The production of acids, resulting from sugar metabolism by these plaque bacteria, and the subsequent drop in plaque pH is the main cause of demineralization of the enamel tooth surface (19).

Current understanding of the microbiology of dental plaque and its relationship to the aetiology of dental caries is mostly gained from data obtained by traditional culture methods. Much of the research to date has suggested that mutans streptococci are the major pathogens involved in the caries process, (for a review of this topic see Tanzer 2001 (71)). Based on available data the *specific plaque hypothesis* was proposed by Loesche and has been easy to promote for the reason that it agrees with the traditional Koch's postulates for infectious diseases in that a specific pathogen could be isolated from the diseased tooth surface, cultured and inserted into a disease-free animal where, given the correct dietary supplements, new lesions would develop. This belief in the pathogenic role of specific organisms in dental plaque initiating caries and continuing the carious process, once a lesion had been initiated, lead to a considerable amount of research into a vaccine for caries (for a review of this topic see Russell, M.V. *et al* 2004 (26)) and, more simply, to the use of microbiological analysis of clinical samples for determining caries risk, complicity with preventive measures and other aspects of clinical cariology. This philosophy has been adopted particularly in the Nordic countries (20, 23, 72).
However, some recent studies indicate that the relationship between caries and *S. mutans* are not absolute. *S. mutans* can persist on tooth surfaces without development of caries and further caries can develop in the absence of *S. mutans* (5, 73).

Examination of the microbiology of dental caries has been hampered by at least two factors. Firstly, the tradition, and clinical usefulness, of focusing on small number of species

thought to be pathogenic and secondly, the lack of useful, rapid identification techniques to evaluate large numbers of bacterial species in large numbers of samples. Over the last two decades advances in methods for bacterial identification have increased our understanding of the dental plaque biofilm and demonstrated that the biofilm composition is probably more complicated and more diverse than expected. Studies using molecular methods have revealed that up to 700 bacterial species can be detected and considered to inhabit the oral cavity. However, only around 50% of this flora is yet cultivable (3-5, 74, 75). If it is assumed that specific organisms may cause dental caries then it must be assumed that some of this uncultivable flora may be partly pathogenic and, therefore, the new molecular methods are needed to investigate these organisms in an attempt to determine the role of these "uncultivables" in dental diseases. Cultivation is, however, still the general practice for detection and enumeration of caries-associated bacteria. The ease of performing presumptive counts of and *Lactobacillus,* compared to the practical difficulty of determining the role of many other species in dental plaque, has also been one factor in retaining the focus on these "key" cariogenic bacteria. It cannot be underestimated, however, how useful simple culture of these two cariogenic organisms is in explaining many aspects of caries risk and caries control. If these organisms are, in fact, surrogate markers of the carious process they have shown particular strength in their association with the disease. This has been shown, for example in several longitudinal studies of caries risk assessment in Iceland (22, 23, 40).

5.2 Clinical diagnosing and sampling method

Earlier studies of the microbiology of fissure plaque have used either "artificial fissures" implanted in occlusal surfaces (33, 76) of pre-existing restoration, or by sampling natural fissures. In the present study the clinical methodology, diagnostic technique and sampling, were

the responsibility of one clinician, Peter Holbrook. It was hoped that this would reduce variation in clinical assessment and sampling. In view of the fact that visual assessment of occlusal surfaces with respect to caries, especially at an early stage, is difficult (77), new diagnostic methods have been developed that, potentially, could increase the accuracy of detecting minor changes in occlusal pits surfaces (78, 79). The reason for this interest is because caries detected at a very early stage can be treated by promoting recalcification of the enamel or by sealing the occlusal surface, rather than restoring the tooth. One such diagnostic tool, the DIAGNOdent® fluorescent probe is said to be very sensitive at detecting early caries. This equipment was being evaluated in the department and its use enabled specimens to be taken from different stages of dental health and early caries. The DIAGNOdent® diagnostic instrument is considered as a clinical support to quantify changes in the physical characteristics of enamel related to demineralization. The method has been used for several years with good result but the reliability and precise mode of action are still being investigated (68, 78). While there is still not total agreement on what the DIAGNOdent® instrument is actually measuring, it is thought to detect porphyrins although their role in tooth destruction is far from clear. This was, nevertheless, not the issue of the present study. The proven diagnostic capability of the DIAGNOdent® instrument was deemed sufficient to enable specimens to be taken at appropriate stages of the disease.

Although this clinical work was a minor part of the project, it was of great importance for the consequent results of the study. The method offers a more sensitive/accurate collection of samples from caries sites. Sampling with paper points, is a method that has mostly been used to sample bacteria from root canals and periodontal pockets (80) where it appears to be a reliable sampling method. The present data demonstrate that it is certainly also a reasonably reliable method for dental plaque sampling from occlusal fissures.

5.3 Cultivation of oral bacteria

Our result showed that *Lactobacillus* sp. was only detected in by cultivation of samples from sites with cavitation. This supports the findings of other studies of their function as secondary pathogens in the caries process but *Lactobacillus* sp. are highly acidogenic and acid tolerant and this supports the conclusion that these bacteria can take advantage of the micro-environment that develops in the tooth fissure as caries proceeds.

In this study, cultivation was performed on non-selective blood agar, both under anaerobic and aerobic conditions and this gave an idea of the total number of viable bacteria in each sample. The cultivable bacterial flora was not identified any further. If the results from cultivation on non-selective media are considered in connection to the microbial diversity of this environment, culture did not provide particularly much information and a non-selective approach to culture of plaque microorganisms clearly does not, realistically, have any part to play in the diagnosis of caries. Selective media clearly can help in providing clinically-meaningful results with respect to caries presence and caries risk but the findings do not, necessarily, relate to the overall changes in dental plaque occurring with the move from health to caries on the tooth surface. The reason for limited numbers of bacteria recovered on blood agar from some of the samples, particularly those from caries-free teeth is uncertain, but a limited number of bacteria in the tooth fissure is one likely explanation and another might be that the transfer of bacteria on to the paper point is less when the tooth fissure is hard and disease free. Saliva samples, which are generally used for enumeration of the classic caries pathogens, probably would have provided: more bacteria; improved recovery of cultivable bacteria on agar plates; and consequently more reliable results from cultivation. However, the aim of the present study was to examine the

bacterial flora of a particular tooth surface and thus mixed samples such as from saliva would not have been representative of the occlusal surface.

5.4 Culture-independent methods

Results from 16S rRNA gene sequencing of several hundred of clones from samples of initial caries and early cavitated sites demonstrate that *S. mutans* was not a dominant species and was not even always detected. Additionally, *Streptococcus sobrinus* that has previously been shown to be particularly prevalent in Iceland (81) was not detected in any of these samples. *Veillonella* species are frequently found in dental plaque associated with health and disease. *Veillonella* are strictly anaerobic Gram-negative cocci that can metabolize lactic acid produced by other bacterial species to form propionic and acetic acids, both weaker acids than lactic acids and less active in demineralization of the tooth enamel. However, in-vitro studies have shown that the combination of *Veillonella* and *S. mutans* allows more acid production and greater demineralization than does *S. mutans* alone (32). *Veillonella* was found in all levels but in elevated numbers in deeper caries in a sample together with *S. mutans* in high proportion as well. One theory is that the interplay of *Veillonella* and *S.mutans* allows the mutans streptococci to metabolise more carbohydrate because the *Veillonella* utilize some of the lactic acid produced and thus raise the pH preventing hindering of further growth of the mutans streptococci. Thus these acid-producing bacteria can continue to exert an influence on the overall properties of the plaque. Then again, confirmation of this theory is difficult were good selective media for *Veillonella* sp. is still not available.

Cultural studies have demonstrated that Gram-positive rods and filaments are commonly found in the mouth and the most numerous group of organisms isolated from dental plaque. This group of bacteria consists of aerobic, facultative anaerobic and strictly anaerobic species such as

Actinomyces sp., *Rothia, Lactobacillus* sp., and *Scardovia* were detected in several samples. *Actinomyces* and *Rothia* were detected in samples in dental plaque on healthy surfaces and initial caries. It has been demonstrated that *Actinomyces species* are predominant at earliest stage in the biofilm formation of teeth (12). These bacteria are also carbohydrate users, but are not powerfully acidogenic or acid tolerant. *Actinomyces* sp. was found on healthy and initial caries tooth surfaces but not in cavitated sites supporting the possible role of *Actinomyces sp*.Three relatively recently described bacteria from dental plaque, *Scardovia, Parascardovia* and *Olsenella profusa* were found in samples from cavitated surface. Lactobacilli were almost only detected in samples from cavitated surface with few exceptions indicating their role in the later process in caries formation. It must be pointed out though, that these bacteria are primarily, making use of a suitable environment and their ability to utilize nutrients that become available for their growth and reproduction. Our results from the 16S rRNA analysis confirm the results of previous studies that Gram-positive bacteria are most commonly found in caries active sites but also show that the diversity decreases with the onset of caries.

5.5 Bacterial diversity

Based on the16S rRNA analyses, the results of this study confirm the diverse microbial community present in the dental plaque on occlusal tooth surfaces. There was a clear trend towards decreasing microbial diversity with increased cavitation. The Simpson diversity index takes into account the number of species present, as well as the abundance of each species. Mainly the results are in agreement with the finding of previous studies, showing the dominance of Gram positive bacteria in the dental plaque associated with dental caries. There was a difference in bacterial composition at the different stages of the caries process. Various *Streptococcus* species dominated in samples from healthy sites (CF) and combined with very

diverse bacteria. In the initial caries samples (CB) a less diverse flora was detected but in samples from cavitated sites (CA), there was a further shift in the flora, towards acid-producing and acid-tolerant bacteria, dominated by *Lactobacillus* sp. The bacterial flora is focusing down to narrower microflora. However, bacterial composition of samples from early cavitated sites is not completely uniform. The findings of this study support that the mixed flora contains organisms with an ability to grow better with the ongoing carious process with lowering pH and changes in the availability and type of nutrients. Thus, dental caries may be a result of a shift in balance of the resident microflora driven by changes in the environment. It can be assumed that certain specific bacterial species are more associated with caries initiation than others but, additionally, a clear subject variation in the bacterial composition was observed in all classes of samples collected.

5.6 Methodological considerations

In theory, amplification efficiency should be the same for all bacteria in a sample if using universal 16S rRNA primer sets and thus a non-selective method. Additionally, only very small quantities of DNA templates are needed for PCR amplification. It should be noted that although this study illustrates the succession quite well towards less diversity with more progressive caries, PCR based methods, such as 16S rRNA and T-RFLP used in this study; do not provide complete information on the relative abundance of bacteria in the plaque but, importantly, it confirms the dominance of bacteria in the sample or environment under investigation and illustrates the bacterial diversity but only misses out uncommon and probably less important species. It would not be cost-effective to analyze and sequence the entire bacterial flora in a sample and generally only a limited number of clones, often around 50 clones per sample, are analyzed. Sequencing 1000 clones, or even more, would be needed to detect the rarest species in

diverse environments and it is important to be aware of these drawbacks of the methodology. It is also possible to calculate the probable coverage of analyzed bacteria of the environment under investigation and use those statistics to predict to some extent on the undetected species of the sample/environment. In microbial studies of environments inhabited by diverse bacteria, unknown, and presumably uncultivable bacteria, molecular methods have many advantages compared to cultivation methods. The sensitivity, the ability to identify the uncultivable in addition to the valuable phylogenic information of predominant microflora, makes 16S rRNA gene sequencing method very attractive for bacterial ecology studies and thus the most applied method in such studies. However, it should be noted that failure to detect cultivable species, such as the well known cariogenic bacteria, *S. mutans* and, possibly, *S. sobrinus*, by 16S rRNA gene analyses does not necessarily mean it is not present in the sample. *S. mutans* is proven to be hard-to-lyse bacteria which could have effect on the available DNA but more likely these species could simply be present in lower numbers than the detection limit of the method, as used in this study, allows. If the aim is to detect and enumerate specific cultivable bacteria, such as a known pathogen, and good selective media is available, this indeed favors cultivation methods. Methods such as 16S rRNA gene sequencing can also facilitate the finding of appropriate media for unknown or uncultivable bacteria. The phylogenic information that 16S rRNA provides are important *per se* but especially concerning the uncultivable bacteria. By constructing phylogenic trees, a comparison of the "uncultivables" to closest cultivable relatives is made possible and, consequently, there are probably more chances of finding the appropriate cultivation media and conditions for many currently uncultivable bacteria. Additionally, it must be one of the ultimate goals of microbial ecology to find, cultivate and describe the appearance and physical characters of new and previously uncultivable bacteria.

T-RFLP analyses that is also based on amplification of 16S rRNA genes has the advantage of being simple and based on producing patterns that are automatically converted into digitized form. The T-RFLP analysis method is, however, not as accurate for bacterial identification as 16S rRNA gene sequencing. It also gives only semi-quantitative data that can function partially for bacterial identification, at least to genus level, if the bacterial composition of given environment is well known. Some researchers have reported problems with incomplete digestion of PCR amplicons, creating artificial T-RFLP pattern (54). To decrease the detection of such artificial peaks in this study, repeated T-RFLP patterns were generated for each sample, as suggested by Dunbar and colleges (82). Additionally, the method can be used in combination with 16S rRNA data from cloning and sequencing and T-RFLP patterns can be compared to these sequences for bacterial identification. There are several other drawbacks of the methodology, such as artificial background noise, making detection of low abundance TRF peaks difficult and reduced accuracy of size determination of fragments. Some investigators have reported that observed TRF fragments are not accurately the same as predicted for any given species and can be anywhere between 0-4 bases longer than predicted by comparison to its 16S sequence (54). Indeed, some of these problems were noticed in this present study. In addition and the greatest disadvantage of the method is due to the many different parameters that affect the pattern analysis such as equipment, analyzing programs, fluorescent primers, restriction enzymes, amount of DNA etc, the comparison to other studies is not practical and almost impossible. However, our data confirms that terminal restriction fragment polymorphism patterns are useful in community analyses up to certain level, especially to show variation in microbial composition of different samples and of different types of environment. The T-RFLP pattern analysis illustrates partially the changes in bacterial composition of the plaque with

changes of the enamel tissue. Such methods could be practical for clinical monitoring of oral microflora in individuals, before and after professional cleaning of dental plaque, and probably more clinically relevant, for monitoring oral microflora before and after antimicrobial treatment of periodontal patient. The terminal restriction fragment length polymorphism method was reasonably consistent with the results of 16S rRNA analyses showing most diversity in samples from healthy tooth surface and decreasing diversity with the onset of caries. It should also be noted that despite the many advantages of 16S rRNA gene analysis, the complications for adoption in routine clinical microbiology should be considered carefully. There have been some valid criticisms on that matter. One is the lack of universally-accepted criteria for 16S rRNA gene sequence-based identification of bacterial isolates is problematic (83). Cost is always a major obstacle in clinical microbiology and is considerably greater than for cultural methods due to specialized equipment, reagents and expertise. Although this cost could be reduced, by purchasing the sequencing service directly, by using public databases, such as GeneBank, for analysis of the sequence data, other problems still arise. The public databases are not peer-reviewed, affecting the quality of available sequence data. This problem especially concerns the need for accurate identification of pathogens.

5.7 Vaccination against specific caries producing bacteria

One important point for consideration following the results of this study is the probability that vaccination against dental caries with a vaccine derived specific from a *S. mutans* serotype c antigen would probably not have been effective. Vaccination against caries has been a long-running project (24-26) and while removal of *S. mutans* serotype c from the mouths of experimental animals (84) and even human subjects (85) has already been demonstrated, there is no certainly that a vaccine would be clinically effective. Vaccines are, indeed, intended for

preventing exogenous organisms entering the body and causing disease. With caries being shown, in this and similar studies, to be an endogenous infection strongly related to particular developments of biofilm ecology (an acidogenic and aciduric dental plaque) it is legitimate to speculate that the role of the bacterium removed from the biofilm (*S. mutans*) would be taken over by another organism and the total activity of the dental plaque biofilm could be the same as it was when *S. mutans* was present. Additionally, there is approximately 50% of the oral flora still uncultivable and the role of those bacteria could possibly have some functional impact on the caries process as well. Taking these findings into consideration and the effort to develop vaccines, specific against mutans streptococci, it is important to study the etiology of all these bacterial species and their potential role in the caries process.

6. CONCLUSIONS

The diagnostic equipment, DIAGNOdent® has recently been developed for the detection of initial caries. This study has shown that by combining this diagnostic technology with traditional microbial cultivation and molecular methods a very effective system can be developed for studying localized bacterial composition in dental plaque. With the more accurate caries detection technology there is a possibility for more precise microbial sampling. Our results, from 16S rRNA gene analysis, confirm the great bacterial diversity present in the dental plaque biofilm but also the decreasing diversity with increased tooth demineralization. The T-RFLP method also illustrates the succession of the dental plaque, showing significant decrease in detected terminal fragments from healthy tooth to early cavitation. This T-RFLP method, which is rather inexpensive, could even provide an alternative for clinical analysis alongside cultivation.

The results of the present study and other recent studies such as by Aas and colleagues 2008 (5), using molecular methods for bacterial identification, indeed broaden the spectrum of bacteria that may be considered cariogenic. However, since 16S rRNA gene analysis is still rather expensive method, screening, diagnosing and disease monitoring large numbers of samples, may be less practical than the use of culture on selective media, as is currently widely adopted. Additionally, though the results of this study support the *ecological plaque hypothesis* and in some way undermine the specificity of certain organisms as the only key etiological agents in caries development, it does not prevent the use of and *Lactobacillus* sp. as surrogate markers for purposes of detection of caries risk and monitoring progress or change the clinical purpose of using these species as indicator organisms in dental caries.

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Takk

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9. APPENDIX

0706 Preliminary investigation of periodontitis using 16S rRNA gene analysis

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The flora of the periodontal pocket in periodontal disease is highly complex. Investigation of the disease-associated changes in flora is difficult and is subject to many uncertainties. Development of molecular biological methods for use in microbial identification has enabled a re-evaluation of the complex flora associated with periodontal disease. Objectives: The aim of this preliminary study was to use culture-independent methods to estimate bacterial diversity in Icelandic subjects. Methods: Samples were collected, from three subjects with chronic adult periodontal disease, by inserting paper points into several periodontal pockets and with a periodontal scaler to scrape one deep pocket. The 16S rRNA genes of the microbial flora in the clinical samples were amplified directly by PCR with bacterial universal primers. PCR products were cloned and sequenced. Results: A total of 373 clones were sequenced, analysed and 62 different species or phylotypes identified from 7 bacterial phyla. Most of the species found are known to be cultivable but several uncultivable phylotypes were found in all three patients; two phylotypes were identified from the newly described TM7 phyla. Most diverse group of species fell into the phylum firmicutes including predominant species as Streptococus constellatus, S. intermedius, Selenomonas sputigena and Peptostreptococcus micros. Putative pathogens such as Fusobaterium nucleatum, Atopobium sp. and Actinomyces sp. were also detected. However, pathogens known to be associated with periodontal disease such as Prevotella sp., Porphorymonas gingivalis and Actinobacillus actinomycetemcomitans were not identified in these samples. Conclusions: In complex microbial communities such as the oral cavity, culture-independent approaches are useful and might reveal undiscovered pathogens. These methods give clues to the composition of the bacterial flora and can be used as a tool to find new putative pathogens associated with the disease. Failure to find the classical cultivable pathogens in these samples should prompt further study.

Seq #76 - J. Microbiology Posters II
PEF of IADR Meeting 15 September 2006 Trinity College Dublin

0126 *S. mutans* from caries-active and caries-free individuals

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A collection of strains of *S. mutans* from caries-active (CA strains) and caries-free individuals (CF strains) has been isolated and investigated for possible differences in pathogenicity. A series of 16 isolates of Strept mutans has been shown previously to differ in adherence to apatite and in release of calcium from apatite on culture (NOF 1997). Objectives: Further studies on this strain collection were performed in order to examine these apparent strain differences in detail. Methods: Bacteriocin-like activity was investigated by stab-inoculation of each of the test strains into pour-plates containing, separately, each of the test strains and 25 laboratory isolates of oral streptococci and other organisms. Electronmicroscopic investigation of CA and CF strains was carried out using negative staining to observe the presence of "fuzzy coat". Immunoelectron microscopy using anti-crude fimbrial antibody and gold labelling was performed to determine possible differences in fimbriation of the CA and CF strains Results: Although CA strains showed more bacteriocin-like inhibition against other mutans streptococci (3.4 vs 1.2 strains; $P<0.01$), the CF strains were more inhibitory to other oral commensals (88/126 vs 59/126 tests; $P<0.001$). Despite considerable strain variation, a clear trend was noted of a thicker "fuzzy coat" layer for the CF strains ($P=0.057$) compared with CA strains. Almost all strains showed gold binding in this outer layer and no clear differences between CA and CF strains were observed. Conclusions: Clear morphological and behavioural differences appear to exist between strains of Strept. mutans although the mechanisms by which these differences affect the cariogenic potential of the strain are not yet clear.

Seq #11 - E. Cariology Poster Session I,
PEF of IADR Meeting 13 September 2006 Trinity College Dublin

237 Changes in Detectable Microbial Flora with the Onset of Caries

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Changes in plaque flora occur as the tooth surface undergoes changes from health through to early caries. Recent developments have enabled more detailed studies of changes in plaque composition and early detection of the carious lesion. Objective: To examine the composition of plaque flora overlying healthy tooth enamel and enamel showing early caries, as detected with the DIAGNODent® apparatus. Methods: Occlusal surfaces of molar teeth were assessed using DIAGNODent®. Paper-point samples from 13 subjects were taken for both culture and bacterial identification using 16S rRNA analysis. Results: Culture yielded a mixed flora that was difficult to elucidate. Use of selective media showed the presence of the well-known putative cariogenic organisms such as mutans streptococci and lactobacilli. 16S rRNA analysis showed a considerable variety of bacterial genera in the plaque. In total, 110 different species/phylotypes were detected from the 576 clones analyzed. Plaque overlying healthy enamel was dominated by various Streptococcus sp. but a broad range of other species was also detected. The flora reduced in variety with initial caries but the composition was not much different from the healthy sites. Further reduction in diversity was seen in samples from early cavitated lesions where Lactobacillus sp. and other acid-tolerant bacteria dominated. Conclusion: The benefits of using molecular methods, compared with culture, for studying the complex oral microbial communities were clearly demonstrated. Data from 16S rRNA gene analysis confirmed the microbial diversity of the plaque biofilm, illustrates the changes in plaque with the onset of early caries and supports the ecological plaque hypothesis in caries.

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