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ABSTRACT

The genus *Actinomyces* consists of a heterogeneous group of gram-positive, mainly facultatively anaerobic or microaerobic rods showing various degrees of branching. In the oral cavity, streptococci and *Actinomyces* form a fundamental component of the indigenous microbiota, being among initial colonizers in polymicrobial biofilms. The significance of the genus *Actinomyces* is based on the capability of species to adhere to surfaces such as on teeth and to co-aggregate with other bacteria. Identification of *Actinomyces* species has mainly been based on only a few biochemical characteristics, such as pigmentation and catalase production, or on the use of a single commercial kit. The limited identification of oral *Actinomyces* isolates to species level has hampered knowledge of their role both in health and disease. In recent years, *Actinomyces* and related organisms have attracted the attention of clinical microbiologists because of a growing awareness of their presence in clinical specimens and their association with disease.

This series of studies aimed to amplify the identification methods for *Actinomyces* species. With the newly developed identification scheme, the age-related occurrence of *Actinomyces* in healthy mouths of infants and their distribution in failed dental implants was investigated. Adhesion of *Actinomyces* species to titanium surfaces processed in various ways was studied *in vitro*.

The results of phenotypic identification methods indicated a relatively low applicability of commercially available test kits for reliable identification within the genus *Actinomyces.* However, in the study of conventional phenotypic methods, it was possible to develop an identification scheme that resulted in accurate differentiation of *Actinomyces* and closely related species, using various different test methods. Genotypic methods based on 16S rRNA sequence analysis of *Actinomyces* proved to be a useful method for genus level identification and further clarified the species level identification with phenotypic methods. The results of the study of infants showed that the isolation frequency of salivary *Actinomyces* species increased according to age: thirty-one percent of the infants at 2 months but 97% at 2 years of age were positive for *Actinomyces*. *A. odontolyticus* was the most prominent *Actinomyces* colonizer during the study period followed in frequency by *A. naeslundii* and *A. viscosus.* In the study of explanted dental implants, *Actinomyces* was the most prevalent bacterial genus, colonizing 94% of the fixtures. Also in the implants *A. odontolyticus* was revealed as the most common *Actinomyces* species. It was present in 84% of

Actinomyces -positive fixtures followed in frequency by *A. naeslundii*, *A. viscosus* and *A. israelii.* In an *in vitro* study of titanium surfaces, different *Actinomyces* species showed variation regarding their adhesion to titanium. Surface roughness as well as albumin coating of titanium had significant effects on adhesion.

The use of improved phenotypic and molecular diagnostic methods increased the accuracy of the identification of the *Actinomyces* to species level. This facilitated an investigation of their occurrence and distribution in oral specimens in both health and disease.

TIIVISTELMÄ

Gram-positiiviset pääosin fakultatiivisesti anaerobiset tai mikroaerobiset *Actinomyces*-bakteerit toimivat streptokokkien ohella primaarivaiheen kolonisoitujina suun eri pehmyt- ja kovakudospinnoille muodostuvissa biofilmeissä. Niiden merkitys kehittyvän biofilmin tärkeänä rakenneosana perustuu ko-aggregaatiokykyyn eri bakteerilajien välillä sekä erityisiin adhesiineihin, joiden avulla ne pystyvät vaikuttamaan tarttumiseen. Aktinomykesten tunnistus kliinisissä laboratorioissa on aiemmin perustunut yksittäisiin biokemiallisiin reaktioihin kuten katalaasiin ja pigmentin muodostukseen. Kiinnostus aktinomykeksiä kohtaan on kuitenkin lisääntynyt viime aikoina huomattavasti, koska *Actinomyces*- lajeja on esiintynyt kliinisissä näytteissä yhä enenevissä määrin ja aiemmasta kirjallisuudesta poiketen niiden on havaittu olevan osallisena myös erilaisissa infektioissa. Viimeaikaisten taksonomisten muutosten myötä *Actinomyces*-lajien tunnnistaminen kliinisissä laboratorioissa on kuitenkin edelleen vaikeutunut huomattavasti. Tässä väitöskirjatyössä kehitetty kattava fenotyyppisiin menetelmiin perustuva identifiointiohjeisto on mahdollistanut eri lajien esiintyvyyden ja merkityksen selvittämisen väitöskirjan muissa osatöissä, joissa selvitimme eri lajien ilmaantumista ja kolonisaation pysyvyyttä vastasyntyneen lapsen suun kehittyvässä bakteeristossa; tutkimme aktinomykesten merkitystä epäonnistuneiden hammasimplanttien pinnan kolonisoitujina ja eri *Actinomyces*-lajien esiintymistä implantin menetykseen johtavassa tulehduksessa; selvitimme *in vitro* -tutkimuksessa tarttuvatko aktinomykekset implanttirakenteen ts. titaanin pintaan, ja voiko tähän tarttuvuuteen vaikuttaa biomateriaalien pinta-ominaisuuksia muuttamalla. Tutkimustulokset osoittivat aktinomykesten esiintyvyyden lapsilla nousevan kolmasosasta jopa 97 %:iin kahden ensimmäisen elinvuoden aikana. Ylivoimaisesti yleisin *Actinomyces*-löydös jokaisella näytteenottokerralla oli *A. odontolyticus*, jota esiintyi yleisesti jo kahden kuukauden ikäisillä lapsilla. *A. naeslundii* oli seuraavaksi yleisin *Actinomyces*-laji, mutta sitä löytyi ensimmäisen kerran vasta vuoden iässä, mikä on mahdollisesti yhteydessä hampaallisuuteen. Tutkimustulosten mukaan aktinomykekset muodostivat yleisimmän bakteeriryhmän (94% näytteistä) epäonnistuneiden implanttien kolonisoitujina. Myös näissä näytteissä *A. odontolyticus* osoittautui yleisimmäksi *Actinomyces*-lajiksi. Lisäksi *A. naeslundii*, *A. viscosus* ja *A. israelii* tunnistettiin yllättävän useista implanttinäytteistä. *In vitro* tutkimus titaanin pintaominaisuuksien vaikutuksesta eri *Actinomyces*-lajien tarttumiseen osoitti, että elektronimikroskoopissa tarkasteltuna eri lajeilla on erilainen affiniteetti titaanin pintaan proteiinipinnoitteen sekä pinnan karheusasteen vaikuttaessa solumääriin.

Tämän väitöskirja-tutkimuksen myötä kehitetyt fenotyyppiset identifiointi-ohjeistot mahdollistavat eri *Actinomyces*-lajien tunnistamisen kliinisistä näytteistä yhä luotettavammin. Luotettava laji-tason identifiointi loi edelleen perustan tutkimukselle, jossa selvitettiin eri *Actinomyces*-lajien osuutta ja merkitystä suun pinnoille muodostuvissa biofilmeissä sekä terveyden että infektioiden kannalta.

Avainsanat: aktinomykes, identifiointi, adheesio, kolonisaatio, implantti, titaani

CONTENTS

ABBREVIATIONS

AHP	Anaerobe Helsinki Positive rod
ATCC	American Type Culture Collection
α-fuc	alpha-fucosidase
β-NAG	beta-N-acetyl-glucosaminidase
β-gal	beta-galactosidase
Bru	brucella blood agar
BSA	bovine serum albumin
CCUG	Culture Collection University of Gothenburg
CFA	cellular fatty acid
CFU	colony forming unit
CFAT	cadmium-fluoride-acriflavin-tellurite
GLC	gas-liquid chromatography
IUCD	intrauterine contraceptive device
ONPG	o-nitrophenyl-β,D-galactoside
PRAS	prereduced anaerobically sterilized
RLB	rabbit laked blood
RNA	ribonucleic acid
PBS	phosphate buffered saline
SEM	scanning electron microscopy
4-MU	4-methyl-umbelliferyl

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Sarkonen N, Könönen E, Summanen P, Könönen M, Jousimies-Somer H. Phenotypic Identification of *Actinomyces* and Closely Related Species Isolated from Human Sources. *J Clin Microbiol* 2001;39:3955-3961.

II Santala A-M, Sarkonen N, Hall V, Carlson P, Jousimies-Somer H, Könönen E. Evaluation of Four Commercial Test Systems for Identification of *Actinomyces* and Some Closely Related Species. *J Clin Microbiol* 2004;42:418-420.

III Sarkonen N, Könönen E, Summanen P, Kanervo A, Takala A, Jousimies-Somer H. Oral Colonization with *Actinomyces* Species in Infants by Two Years of Age. *J Dent Res* 2000;79:864-867.

IV Sarkonen N, Könönen E, Eerola E, Könönen M, Jousimies-Somer H, Laine P. Characterization of *Actinomyces* Species Isolated from Failed Dental Implant Fixtures. *Anaerobe* 2005;11:231-237.

V Sarkonen N, Könönen E, Lounatmaa K, Könönen M. Effect of Different Titanium Surfaces on *Actinomyces* Adhesion: *In Vitro* Electron Microscopy Study. Submitted.

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In addition, the thesis includes unpublished results.

1 INTRODUCTION

Actinomyces are commonly found in considerable proportions in indigenous microbiota of the mouth, and they are an important group of early colonizers in dental plaque formation (Nyvad and Kilian 1987; Liljemark *et al.* 1993; Könönen *et al.* 1999). The composition of the mature dental plaque, which often includes potential oral pathogens, is therefore dependent on the primary binding of these pioneer bacteria. The time of appearance and nature of species that are involved in the primary colonization are of great importance as they form the basis for further colonization, and therefore, may substantially affect the composition of the developing, both indigenous and pathogenic, oral microbiota. The significance of the species is based on their capacity to adhere to deposits as well as their co-aggregating properties with other bacteria (Ellen and Sivendra 1985; Gibbons and Hay 1988; Cisar *et al.* 1989). Certain *Streptococcus* and *Actinomyces* species predominate in early plaque formation and colonize first on tooth and/or mucosal surfaces providing a substrate for the adherence of more fastidious plaque microbes, including gram-negative, strictly anaerobic bacteria (Kolenbrander 1993; Nesbitt *et al.* 1993).

Actinomyces may be isolated from infections in the oral region as well as from other sites of the human body (Schaal 1986). Knowledge of the interrelationships among *Actinomyces* and related organisms has improved greatly in the past decade, and the use of improved phenotypic and molecular diagnostic methods of analysis has not only resulted in more reliable species identification but has also facilitated the recognition of many new taxa (Funke *et al.* 1994; Ramos *et al.* 1997; Hall *et al.* 2002; Hall *et al.* 2003; Hall *et al.* 2003; Woo *et al.* 2003). However, little effort has been directed to study the establishment and distribution of different *Actinomyces* species in the healthy oral cavity. Also, the identification of *Actinomyces* species isolated from oral infections has mainly been based on only a few biochemical characteristics, such as pigmentation and catalase production, or on the use of a single commercial kit. This somewhat unreliable identification of oral *Actinomyces* isolates to species level has hampered knowledge of their relationships, natural habitats, prevalence and pathogenicity (Liljemark and Bloomquist 1996).

2 REVIEW OF THE LITERATURE

2.1 Oral bacterial colonization

In the mouth, which is an open-growth system, bacteria have to attach to a surface in order to persist and grow (Gibbons and van Houte 1975). Attachment is an essential step for colonization and the process among bacteria occurs in a highly selective manner; different species among genus, e.g. *Streptococcus*, attach better to tongue and/or cheek surfaces, whereas others prefer non-shedding tooth surfaces (Frandsen *et al.* 1991). However, not all of the micro-organisms that enter the mouth are able to colonize. Most bacteria in the oral cavity are only transient, while others succeed in finding a suitable surface for persistent colonization. The microbiota consists of commensals and, on some occasions, also pathogens. The commensal microbiota is beneficial to the host by blocking adherence and metabolism of pathogens. However, members of the commensal microbiota can act as opportunistic pathogens when their habitat is disturbed or when micro-organisms are found at sites not normally accessible to them. It has been suggested that dental diseases are caused by imbalances in this resident oral microbiota (Marsh 2003).

2.1.1 The oral cavity as a habitat for bacteria

Several distinct surfaces in the mouth provide different habitats for bacteria to colonize. The oral biofilm ecology is constantly changing: potential niches increase significantly when tooth eruption allows further microbial colonization. Moreover, the development from the primary to permanent dentition, possible loss of teeth, and use of prosthetic reconstructions continuously changes the ecological conditions in the oral cavity, making it a unique habitat for colonization. All these surfaces are bathed by oral fluids: saliva and gingival crevicular fluid serve nutrients and deliver components of host defences for regulating the colonization. They also help remove loosely attached bacteria from surfaces. Fluctuations in the stable oral ecosystem can further be induced by external factors, such as daily food, dental care or possible antibiotic therapy.

2.1.2 Pellicle formation and initial bacterial adhesion

All exposed surfaces in the oral cavity are immediately coated with a proteinaceous layer providing receptor sites for bacterial adhesion (Gristina 1987). This acquired pellicle is a bacteria-free biofilm, covering oral hard and soft tissues as well as non-oral surfaces (Dawes 1963; Lendenmann *et al.* 2000). The major salivary proteins in pellicle are acidic proline-rich protein, statherin, secretory IgA, cystatin, mucin, lactoferrin, lysozyme, and amylase (Lendenmann *et al.* 2000). Early colonizers, such as streptococci and *Actinomyces,* are able to recognize these proteinacous components (Kolenbrander and London 1993), and therefore the pellicle determines at least the initial bacterial attachment.

The simultaneous presence of bacterial adhesion and inhibition factors indicates that the pellicle functions in recruiting micro-organisms and at the same time controls their growth, thereby dictating the final microbial profiles on pellicle (Clark *et al.* 1978; Gibbons and Hay 1989; Lendenmann *et al.* 2000; Li *et al.* 2004). The composition of this pellicle varies in different parts of dentition (Carlen *et al.* 1998): different target surface (e.g. biomaterial) properties may also influence different pellicle compositions and, therefore, the initial plaque formation (Leonhardt *et al.* 1995).

2.1.3 Bacterial attachment and coaggregation

When bacteria are transported towards a surface they are capable of contact with it. Adhesion usually takes place through adhesins, located on bacterial surface, or fimbriae, which further interact with specific target molecules or ligands (Ellen *et al.* 1997). After this initial adhesion to a surface, more physical anchorage called attachment can occur. Attachment is an essential step for further colonization. When bacteria have been firmly attached they start to grow and communicate, and therefore the biofilms can further develop (Quirynen and Bollen 1995; Costerton 1999). Coaggregation is a process where distinct bacteria in suspension (in the development of multi-species biofilms) attach to one another with specific molecules (Whittaker *et al.* 1996; Rickard *et al.* 2003). In coaggregation, bacterial strains bind specifically to other bacteria. Each species seems to have specific partners, such as *Streptococcus* sp. or *Porphyromonas gingivalis* with *Actinomyces naeslundii* and *Prevotella loescheii* with *Actinomyces israelii* (Kolenbrander 1988). Also, certain *Capnocytophaga* strains coaggregate with strains of *Actinomyces israelii* but not with *Actinomyces viscosus* or with any streptococci (Kolenbrander and Phucas 1984; Kolenbrander and Andersen 1986) suggesting co-adhesion between pairs being highly species depending. Interactions between different bacteria and between bacteria and their host are factors which control the balance within the microbiota and the development of possible pathologic conditions (Kolenbrander 2000).

2.2 *Actinomyces* in humans

2.2.1 Natural habitat

Many *Actinomyces* species and related organisms belong to anaerobic indigenous microbiota of human mucous membranes of the oropharynx, gastrointestinal tract and female genital tract. Members of the genus belong to the initial colonizers on oral surfaces, contributing to oral biofilm development at early stages (Socransky 1970; Socransky *et al.* 1977; Theilade *et al.* 1982; Nyvad and Kilian 1987; Gibbons 1989; Marsh and Martin 1992). Despite the plethora of new species defined from human sources in recent years, it is clear that knowledge of the habitats, clinical prevalence, and pathogenic potential of many *Actinomyces* and related organisms is inadequate, and

there are indications that much new diversity still remains to be discovered (Hall *et al.* 1999; Hall *et al.* 2001).

2.2.2 Actinomyces in infections

Though *Actinomyces* species are part of the normal, resident microbiota of distinct surfaces of the mouth, they contribute to different plaque-involved diseases, e.g. dental caries and periodontal diseases (Moore and Moore 1994). *Actinomyces* are commonly found in endodontic infections as part of a polymicrobial infection (Peters *et al.* 2002). Although *Actinomyces* species have been associated with teeth with necrotic pulps, they are mainly connected with cases of failed root canal treatment (Byström *et al.* 1987; Sundqvist *et al.* 1998; Kalfas *et al.* 2001; Xia and Baumgartner 2003). *Actinomyces* are capable of causing extraradicular infections, thus preventing periapical healing by conventional therapy (Happonen *et al.* 1985). Members of the genus have also frequently been isolated from infected dentin of active root caries lesions (van Houte *et al.* 1994; Brailsford *et al.* 1999; Shen *et al.* 2005). Although especially *A. naeslundii, A. israelii,* and *A. gerencseriae* have been the most isolated species, the role of individual *Actinomyces* species in the root caries process remains unclear (Brailsford *et al.* 1999). Also, various reports of prevalence of *Actinomyces* species in periodontal health and disease have been published. However, the subgingival actinomycetal microbiota (with high detection frequencies up to 98%) of both chronic periodontitis patients and periodontally healthy patients has been reported to be very similar (Ximenez-Fyvie *et al.* 2006).

In recent years, *Actinomyces* and related organisms have attracted the attention of clinical microbiologists, mainly because of a growing awareness of their presence in clinical specimens and because of their association with disease (Funke *et al.* 1997; Hall *et al.* 1999; Hall *et al.* 2001). In addition to various oral infections, actinomycetes can act as major pathogens leading to extra-oral actinomycosis in different parts of the human body characterized by abscess formation, tissue fibrosis and draining sinuses (Schaal and Lee 1992). All the *Actinomyces* species etiologically involved in actinomycotic lesions in humans belong to the indigenous facultatively pathogenic microbiota of the human mucosal surfaces, especially in the oral cavity. *A. israelii* and *A. gerencseriae* are, by far, the most important agents of this disease (Schaal and Lee 1992; Pulverer *et al.* 2003); however, some newer species, including *A. turicensis* and *A. europaeus,* are also clearly associated with mixed infections in superficial soft tissue abscesses (Sabbe *et al.* 1999). These pathogenic species are rarely isolated alone, but rather as part of polymicrobial infection together with various aerobic and/or anaerobic species.

Several *Actinomyces* species, including *A. cardiffensis* (Hall *et al.* 2002), may be involved in pelvic inflammatory disease associated with intrauterine contraceptive devices (Yoonessi *et al.* 1985; Evans 1993; Woo *et al.* 2002; Hall *et al.* 2003). Other species, e.g., *A. meyeri,* have been isolated from infections associated with endoscopic stents in chronic pancreatitis (Harsch *et al.* 2001) as well as from pyogenic liver abscesses (Miyamoto and Fang 1993; Felekouras *et al.* 2004). *A. naeslundii* and *A. israelii* are known to avidly adhere to hip joint

prosthesis to cause infection (Hall *et al.* 1999; Zaman *et al.* 2002). However, the natural habitat of many *Actinomyces* species has remained obscure and their clinical relevance as a part of a polymicrobial infection is not fully understood. Also, infections due to *Actinomyces* can be underdiagnosed because the identification to the species level can be notoriously difficult and time-consuming (Sabbe *et al.* 1999; Hall *et al.* 2001). In addition, there are a number of species differences in antimicrobial suspectibility profiles which suggests that the accurate identification and specification may have an impact on clinical outcome (Smith *et al.* 2005).

2.3 Taxonomy and identification of *Actinomyces*

2.3.1 Taxonomy

The highest rank in bacterial classification is domain which is followed by phylum, class, subclass, order, suborder, family, genus, and species (Stackebrandt *et al.* 1997). These taxonomic ranks and examples are depicted in Table 1. Families share phenotypic characteristics which should be consistent phylogenetically. *Actinomyces* belong to the family *Actinomycetaceae* (Buchanan 1918). Other genera in this family are *Actinobaculum, Arcanobacterium, Mobiluncus,* and *Varibaculum.* All species within a genus form a well-defined group, clearly separated from other genera. The genus *Actinomyces* was first named by Harz in 1877, when he described a causative agent of bovine actinomycosis. The species (e.g. *A. israelii, A. odontolyticus*) is the basic and the most important group in bacterial systematics. Strains are defined as the descendants of a single isolate in pure culture, and usually are made up of a succession of cultures ultimately derived from an initial single colony. A type strain is one specific representative strain selected to describe a species (Stackebrandt *et al.* 1997).

Table 1. Taxonomic ranks

Formal rank	Example
Domain	Bacteria
Phylum	*Actinobacteria*
Class	*Actinobacteria*
Subclass	*Actinobacteridae*
Order	*Actinomycetales*
Suborder	*Actinomycineae*
Family	*Actinomycetaceae*
Genus	*Actinomyces*
Species	*Actinomyces odontolyticus* (example)

Adapted and modified after Stackebrandt *et al.* (1997).

During the past ten years the taxonomy of *Actinomyces* has undergone considerable changes and expansions, with a plethora of new species defined (Table 2). The novel species include *A. cardiffensis* (Hall *et al.* 2002), *A. hongkongensis* from pelvic actinomycosis (Woo *et al.* 2003), *A. nasicola* from nose (Hall *et al.* 2003), *A. dentalis*

(Hall *et al.* 2005), and *A. oricola* from dental abscess (Hall *et al.* 2003). Also, some *Actinomyces*-like isolates have been classified as *Varibaculum* sp. (Hall *et al.* 2003) and few former *Actinomyces* species have been assigned to the closely related genera *Arcanobacterium* and *Actinobaculum* (Lawson *et al.* 1997; Ramos *et al.* 1997). In addition, Johnson *et al.* (1990) have proposed subdivision of *A. naeslundii* into three new genospecies: genospecies 1 included *A. naeslundii* serotype I; genospecies 2 included *A. naeslundii* serotype II and III, and human strains of *A. viscosus* (serotype II); genospecies 3 comprised *Actinomyces* WVA 963. In our study, however, we have kept *A. naeslundii* and *A. viscosus* as separate species based on their biochemical key reactions (*A. naeslundii* is catalase-negative and urease- positive whereas *A. viscosus* is catalase-positive and urease-negative).

TABLE 2. Current *Actinomyces* and closely related genera from human sources.

Current name	Previous nomenclature /Taxonomic Position	Original isolation site	Reference
Actinomyces cardiffensis	New species	Pleural fluid, brain, jaw, IUCD	Hall *et al.* 2002
Actinomyces dentalis	New species	Dental abscess	Hall *et al.* 2005
Actinomyces europaeus	New species	Human abscesses	Funke *et al* 1997
Actinomyces funkei	New species	Blood	Lawson *et al.* 2001
Actinomyces georgiae	*Actinomyces* DO8	Periodontal flora	Johnson *et al.* 1990
Actinomyces gerencseriae	*Actinomyces israelii* serotype II	Periodontal flora	Johnson *et al.* 1990
Actinomyces graevenitzii	New species	Respiratory tract	Pascual Ramos *et al.* 1997
Actinomyces hongkongensis	New species	Polymicrobial infection	Woo *et al.* 2003
Actinomyces israelii	--	Tonsillar crypts, dental plaque, human actinomycoses	Brock *et al.* 1969 (<Kruse 1896)
Actinomyces meyeri	--	Periodontal sulcus, cervicofacial abscesses	Cato *et al.* 1984 (<Prevot 1938)
Actinomyces naeslundii genospecies 1	--	Oral cavity, dental plaque, cervicovaginal secretions	Thompson and Lovestedt 1951
Actinomyces nasicola	New species	Antrum aspirate	Hall *et al.* 2003
Actinomyces neuii ssp. *neuii*	CDC Group 1-like Coryneform	Abscess, blood	Funke *et al.* 1994
Actinomyces neuii ssp. *anitratus*	CDC Group 1 Coryneform	Abscess, blood	Funke *et al.* 1994
Actinomyces odontolyticus	--	Deep carious lesions	Batty 1958
Actinomyces oricola	New species	Dental abscess	Hall *et al.* 2003
Actinomyces radicidentis	New species	Oral	Collins *et al.* 2000
Actinomyces urogenitalis	New species	Urogenital	Nikolaitchouk *et al.* 2000
Actinomyces naeslundii genospecies 2	*A. viscosus*--	Human actinomycoses, eye infections, periodontal diseases	Gerencser *et al.* 1969
Actinobaculum schaalii	New species	Blood	Lawson *et al.* 1997
Actinobaculum urinale	New species	Urine	Hall *et al.* 2003
Arcanobacterium bernardiae	*Actinomyces bernardiae*	Abscess/blood	Pascual Ramos *et al* 1999
Arcanobacterium pyogenes	*Actinomyces pyogenes*	Polymicrobial infection	Pascual Ramos *et al* 1999
Varibaculum cambriensis	New species	Polymicrobial infection, abscess	Hall *et al.* 2003

2.3.2 Conventional identification

Traditionally, descriptive and diagnostic bacteriology has been based on phenotypic characteristics of bacteria and their function. The presumptive identification of *Actinomyces* is based on colony and cell morphology and growth patterns in different gaseous athmospheres (aerotolerance). *Actinomyces* strains produce variable colony forms from regular smooth to irregular rough, or crumbled colonies (Schaal 1986). Certain species have unique colony characteristics: *A. odontolyticus* produces red-brown pigment on blood agar and *A. israelii* colonies are white, irregular, and "molar-tooth"-shaped. The genus cannot be regarded as strictly anaerobic but rather facultatively anaerobic or microaerobic (Slack and Gerencser 1975). *Actinomyces* species ferment glucose to a characteristic pattern of metabolic end products consisting of succinic, lactic, and acetic acids.

The genus consists of a heterogeneous group of gram-positive, straight or slightly curved rods but some species may even appear as short coccobacilli. As they are often pleomorphic in shape, some cells show true branching, while others, such as *A. israelii,* can be filamentous. Production of catalase and indole, reduction of nitrate, and synergistic hemolysis (the CAMP test) are used in presumptive identification of the genus. For a precise identification to the species level, a wide battery of tests are needed, as described in detail in anaerobic bacteriology manuals (Summanen *et al.* 1993; Jousimies-Somer *et al.* 2002).

2.3.3 Molecular methods for identification

Nucleotide sequence analysis of the bacterial 16S rRNA gene has greatly expanded the ability to reliably identify bacterial isolates to the species level (Woese 1987). Thousands of rRNA sequences are currently electronically freely available by multiple gene banks. Diagnosis of pelvic actinomycosis has recently been confirmed in several studies based on sequencing (Woo *et al.* 2002; Elsayed *et al.* 2006). However, evaluation of gene sequence analysis for the identification of *Actinomyces* species compared to other methods in clinical laboratories is still controversial. Checkerboard DNA-DNA hybridization has been used for the detection and identification of *Actinomyces* species and genospecies from plaque samples (Ximenez-Fyvie *et al.* 1999). Hall *et al*. (1999) have shown that amplified 16S ribosomal DNA restriction analysis (ARDRA) is a rapid and highly discriminatory method for identification of clinical *Actinomyces* isolates. However, it only seems to be suitable for specialist laboratories. Pyrolysis mass spectrometry (PMS) has been used in differentiating groups of organisms but seems to be impractical for the identification of *Actinomyces* isolates (Hall *et al.* 1999).

2.4 *Actinomyces* in oral biofilms

2.4.1 Adhesion mechanisms of Actinomyces

Actinomyces species seem to display different patterns of colonization and distribution in the mouth, which may be correlated with their different affinities and binding specificities to distinct surfaces (Ellen 1976; Cisar *et al.* 1984). Adhesins mediate the attachment of *Actinomyces* to host cells but also to other bacteria involving intra- or intergeneric coaggregation. Some *Actinomyces* species, such as *A. naeslundii* and *A. viscosus*, have a superior ability to adhere to a surface with their special surface structures, type 1 and 2 fimbriae (Cisar *et al.* 1984; Cisar *et al.* 1989). Type 1 fimbriae (present mainly on *A. viscosus*) have affinity for proline-rich proteins and protein statherin on collagen and tooth enamel (Gibbons and Hay 1988; Gibbons and Hay 1989; Liu *et al.* 1991) through adhesin-receptor binding, while type 2 fimbriae (present on *A. naeslundii* and *A. viscosus*) bind to epithelial and bacterial surfaces (Cisar *et al.* 1984; Strömberg *et al.* 1992; Hallberg *et al.* 1998). Type 2 fimbrial lectins of *A. naeslundii* and *A. viscosus* facilitate phagocytosis by recognizing the lactose-containing receptors on polymorphonuclear leukocytes (Sandberg et al. 1986). Other *Actinomyces* species, e.g., *A. israelii*, *A. meyeri,* and *A. gerencseriae,* are reported to lack these kinds of fimbriae and related genes (Cisar *et al.* 1983; Yeung 1992). *A. odontolyticus*, on the other hand, exhibits a genetically related but functionally distinct adhesin, structurally different from fimbriae (Cisar *et al.* 1983; Hallberg *et al.* 1998). Different surface-associated molecules on *Actinomyces* cells have been suggested to determine, to a large extent, the ecological niche of individual species within the oral cavity (Hallberg *et al.* 1998). In addition, the receptors on *Actinomyces* cells can be recognized by adhesins of other bacteria in intergeneric coaggregation (Ruhl *et al.* 2004).

2.4.2. Oral colonization in infants – mucosal surface

At birth, the oral cavity is sterile and composed solely of the soft tissues. Acquisition of the resident oral microbiota begins within first few hours. The mouth becomes colonized from the environment, first from the birth canal and then from the mother during the first feeding. Bacteria that are capable of attaching to epithelial cells or co-aggregating with other species attached to these surfaces have the advantage of colonizing mucosal surfaces (van Houte 1983). For a long time, anaerobes were considered to be rare in infants' mouths as they were assumed to be dependent on the environment of gingival crevices and therefore on the presence of teeth (Socransky and Manganiello 1971; Evaldson *et al.* 1982). However, according to studies by Könönen *et al.* (1992; 1999), initial colonization with anaerobes occurs within the first three months of life and anaerobes are frequently isolated from the edentulous mouths of infants. Bacterial colonization of the oral cavity seems to be a selective process relative to the age of infants: different species colonize at appropriate times for them (Könönen 2000). The colonization patterns of distinct *Actinomyces* species to oral surfaces have also been shown to differ (Ellen and Sivendra 1985).

2.4.3 Dental plaque – tooth surface

Dental plaque accumulation starts within few hours of tooth eruption with the early colonization of streptococci and *Actinomyces* species on pellicle-coated tooth surfaces (Socransky *et al.* 1977; Nyvad and Kilian 1987; Kolenbrander *et al.* 1990). Bacterial surface molecules interact with components of the acquired pellicle, enabling the bacteria to attach to the pellicle-coated tooth surface despite the mechanical shearing forces of salivary flow. The early colonizers are of great importance because they provide attachment substrates and thus influence the succeeding stages of plaque biofilm formation via coadhesion properties (Kolenbrander 1989; Goulbourne and Ellen 1991; Nesbitt *et al.* 1993). The ability of *Actinomyces* species of both intra- and intergeneric coaggregation facilitates their accumulation in early plaque (Marsh 2004). These later colonizers prefer to attach to the performed layer of early-colonizing bacteria rather than the saliva-coated tooth surface. It has been recognized that bacteria exist in complexes in developed plaque (Socransky *et al.* 1998). This indicates that there is a degree of order in colonization of different bacteria. For example, as gingivitis develops, a key change in the microbial composition of dental plaque is the ascendancy of *Actinomyces* spp. and gram-negative rods at the expense of *Streptococcus* spp. (Dalwai *et al.* 2006).

2.4.4 Bacterial adhesion to biomaterials – inorganic surface

Biomaterials are used extensively in medicine and dentistry, e.g. in prosthetic hip joints, catheters, and dental implants. Defined as biocompatible materials, they are mostly metals, ceramics, and polymers, which are used in living tissue or in contact with the tissue to replace diseased or damaged tissues. Because bacterial infection of biomaterials is the most important factor for failure of these medical devices (Razatos *et al.* 1998), initial bacterial adhesion and further development of complex multibacterial biofilms on biomaterial surfaces is a common clinical problem (Costerton *et al.* 1999; Drake *et al.* 1999). On the other hand, prevention of bacterial adhesion, for example by bacteriocins, have been used to decrease bacterial attachment to medical devices; this may represent a novel strategy to control catheter-related infections (Levy 2004; Fontana *et al.* 2006). However, antibiotic catheters may be at a higher risk of being colonized by antibiotic-resistant bacteria (Tambe *et al.* 2001).

Adhesion of bacteria to solid surfaces depends on many factors, including bacterial characteristics, environmental factors (e.g., the presence of serum proteins), and material properties of the target surface (Mabboux *et al.* 2004). For a given material surface, different bacterial species and strains adhere differently; this can be explained by their different physicochemical characteristics. Roughening increases the area available for adhesion, serving pits and grooves, and therefore providing favorable sites for colonization (Brecx *et al.* 1983; Nakazato *et al.* 1989). Although rough surfaces harbor more plaque than smooth surfaces (Smales 1981; Bollen *et al.* 1996), it is still unclear whether all early-colonizing species prefer rough and irregular surfaces for initial adhesion. Different prostheses or implant devices have different surface roughnesses or finishes which may play a role in bacterial attachment and initiation of biomaterial-related

infection (An *et al.* 1997). The adhesion of *Actinomyces* cells to a solid surface, such as intrauterine contraceptive device and prosthetic hip joint, is implicated in the infectious processes of these non-oral sites (Yoonessi *et al.* 1985; Hall *et al.* 1999; Zaman *et al.* 2002). Similarly, osseointegrated dental implants provide hard, non-shedding surfaces for microbial plaque accumulation, including *Actinomyces* (Nakou *et al.* 1987; Mombelli *et al.* 1988; Rutar *et al.* 2001).

2.5 Dental implants

2.5.1 Terminology

Dental implant is a permucosal device which is biocompatible and biofunctional and is placed on or within the bone associated with oral cavity to provide support for fixed or removable prosthetics. The implant is composed of a fixture which is placed surgically in the jaw bone and is allowed to bond with the bone and serve as an anchor for the replacement of a tooth or teeth. Histologically these structures possess no supporting structure like periodontal ligament. The abutment is an attachment placed on top of the fixture. Implant denture receives its stability and retention from a dental implant and is fitted over the abutment(s). When this system is under masticatory force, the implant is "loaded". Osseointegration implies that a contact is established without interposition of non-bone tissue between normal remodeled bone and an implant entailing a sustained transfer and distribution of load from the implant to and within the bone tissue at light microscopy level (Brånemark 1985). Implants are therefore closely linked to oral tissues, which signify that plaque control is of greater importance for implants than for natural teeth (Rams *et al.* 1984). Without the initial attachment to implant surfaces by early colonizers, subsequent polymicrobial accumulation and colonization leading to implant-associated infections do not occur.

2.5.2 Titanium as a biomaterial

Titanium is the most common implant biomaterial used to replace missing teeth because of its excellent corrosion resistance combined with an exceptional degree of biocompatibility in the oral environment. Titanium surface is very reactive mainly due to the surface oxide layer covering it (Kasemo and Lausmaa 1988). Titanium adsorbs proteins, such as albumin, collagenase, fibronectin, and fibrinogen from oral biological fluids (Kane *et al.* 1994; Steinemann 1998). However, the importance of these specific proteins or protein films on the characteristics of titanium is not generally accepted (Serro *et al.* 1997; Serro *et al.* 1999; Serro *et al.* 2004). Different surface properties of titanium are able to influence the formation of bone-implant contact. Rough surfaces seem to favor bone deposition, offering better bone anchorage compared to smooth titanium surface (Wennerberg *et al.* 1995). Roughness of titanium surfaces are also known to play a predominant role in bacterial adhesion and plaque rate (Quirynen *et al.* 1996).

2.5.3 Implant therapy

Osseointegrated (osseointegration named by Brånemark in 1952) dental implants form the major tool in prosthodontics to replace teeth ranging from a single tooth to full dentures (Weber and Lang 1991). Although most implants are extremely successful with survival rates up to 98% (Adell *et al.* 1981; Albrektsson *et al.* 1986; Melo *et al.* 2006), implants can fail. Two main reasons for this failure are bacterial infection and mechanical stress (Becker *et al.* 1990).

2.5.4 Implant failure

One of the key factors for the success of dental implants is the maintenance of healthy tissues around them. Bacterial plaque accumulation induces inflammatory changes in the soft tissues surrounding oral implants and may lead to progressive destruction (peri-implantitis) and, ultimately, to implant failure. Implant failure has been defined as the inadequacy of the host tissue to establish or to maintain osseointegration (Esposito *et al.* 1998). Implant failures have been classically ascribed as early and late failures. Early implant failures include improper preparation resulting in bone damage, bacterial contamination, improper surgical placement, and premature fixture overload (Rosenberg *et al.* 1991). The so-called late implant failures may be due to peri-implantitis or biochemical overload, or a combination of these two occurring in situations where osseointegration of previously stable implant is lost (Tonetti 2000). Peri-implantitis is a site-specific infection resulting in loss of supporting bone around an osseointegrated implant (Mombelli *et al.* 1987).

2.5.5 Microbiology related to implants

Similar to natural teeth, dental implants provide a surface for microbial colonization (O' Mahony *et al.* 2000). Investigations of the microbiota related to implants have shown that both sub- and supragingival bacteria on implants are originally derived from the natural flora of the oral cavity (Heimdahl *et al.* 1983; Lekholm *et al.* 1986). Therefore, the status of remaining dentition reflects the microbiota on these implant sites. For example, dentate patients are known to have more periodontal pathogens at their implant surfaces compared to edentulous implant patients. This suggests that periodontal pockets provide a reservoir for colonization of these sites (Apse *et al.* 1989; Quirynen and Listgarten 1990). Opportunistic periodontal pathogens, such as *Actinobacillus* (currently *Aggregatibacter*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia,* and *Fusobacterium nucleatum,* have been intensively investigated in association with peri-implantitis and failing implants (Leonhardt *et al.* 1999; van Winkelhoff *et al.* 2000). On the other hand a later study by Leonhardt *et al.* (2002) showed that the presence of these periodontal species may not be associated with impaired implant conditions but rather with being part of the resident microbiota. Predominant bacteria around periodontally healthy teeth and implants, however, are reported to be very similar (Apse *et al.* 1989; Mombelli and Mericske-Stern 1990).

3 WORKING HYPOTHESES AND AIMS OF THE STUDY

Working hypotheses:

1) *Actinomyce*s isolated from human sources can be reliably identified to species level by use of phenotypic methods.

2) *Actinomyces* species will establish infants' oral cavity during the first year of life.

3) Different *Actinomyces* species will have different susceptible times for their colonization in infants' mouths.

4) Certain *Actinomyces* species will be connected to infections associated with failed dental implants.

5) Differently processed titanium surfaces *in vitro* may have effect on the adhesion of distinct *Actinomyces* species.

The aims of the present study were:

1) to clarify the phenotypic and molecular identification methods of *Actinomyces* and to compare different methods used in clinical microbiology laboratories

2) to examine, using a longitudinal study design, the age-related occurrence and distribution of oral *Actinomyces* species in infants prior to tooth eruption and in children with erupted teeth

3) to investigate the occurrence and distribution of different *Actinomyces* species in explanted fixture specimens in order to clarify the involvement of *Actinomyces* species in oral implant-related infections

4) to examine the impact of titanium surfaces processed in various ways on initial adhesion of *Actinomyces* species and to evaluate the effect of albumin coating on this adhesion

4 MATERIALS AND METHODS

4.1 Clinical isolates and reference strains (study I-V, unpublished)

The bacteria used in Studies I-VI are depicted in Table 3. The clinical isolates were obtained from infants' saliva, failed dental implant fixtures, submandibular abscesses, and from non-oral sites. Strains exhibiting gram-positive (often branching) rod-shaped morphology were presumptively assigned as members of the genus *Actinomyces* based on suspectibility to special antimicrobial potency disks vancomycin and kanamycin and resistance to colistin, and on production of succinic acid as the major end product of glucose metabolism as determined by GLC. Twenty-one reference strains from international culture collections (ATCC, CCUG) were used as control strains in developing the identification scheme. All strains used in the studies were revived from frozen (-70°C) stocks, subcultured on Brucella blood agar twice, and incubated anaerobically at 35-37 ^{o}C for 3-5 days before testing.

4.2 Identification methods (study I-IV, unpublished)

4.2.1 Biochemical characterization

Colony morphology was examined under dissecting microscope and cell morphology in Gram stained preparations. Pigmentation was assessed on Brucella and rabbit laked blood (RLB) agar media after incubation for 4-5 days, production of catalase was tested using 15% hydrogen peroxide (H_2O_2) and reduction of nitrate to nitrite using a disk test (Jousimies-Somer *et al.* 2002). *Staphylococcus aureus* ATCC 25923 was used as an indicator strain for the CAMP test (synergistic hemolysis) on Brucella blood agar. Enzyme tests were performed using individual diagnostic tablets (Rosco, Taastrup, Denmark), including hydrolysis of urea and esculin, and production of α-fucosidase, α-glucosidase, β-galactosidase (ONPG), β-N-acetyl- glucosaminidase, α-mannosidase, and arginine dihydrolase (the latter two only for *A. israelii* and *A. gerencseriae*), l-arabinose, and β-xylosidase (for differentiation of *Arcanobacterium bernardiae* and *Actinomyces turicensis*) with incubation at 35-37°C for 4 h in air according to the manufacturer's instructions. The fermentation of carbohydrates, including arabinose, glucose, maltose, mannitol, raffinose, rhamnose, sucrose, trehalose, and xylose, were tested using prereduced anaerobically sterilized (PRAS) biochemical media incubated at 35-37°C for a minimum of 5 days.

Table 3. Subjects, strains and identification methods included in Studies I-V, unpublished

Study	Subjects	Bacterial strains	Source	Identification methods used
I	---	19 reference strains	ATCC, CCUG (n=19)	Phenotypic testing:developed identification scheme
		70 clinical isolates	Infectious origin; (n=41) Human saliva; (n=29)	API ZYM (bioMérieux, Marcy-l´ Etoile, France)
				4-MU linked substrates (SIGMA, St. Louis, USA)
II	---	21 reference strains	ATCC, CCUG (n=21)	
		86 clinical isolates	infectious origin; (n=65) human resident flora; (n=21)	RapID ANA II (Remel, Lenexa, Kans., USA)
				Rapid ID 32 A (bioMérieux, Marcy-l´ Etoile, France)
				Rapid CB Plus (bioMérieux, Marcy-l´ Etoile, France)
				BBL Crystal ANR ID (Becton Dickinson Microbiology System, Cockeysville, Md., USA)
III	39 infants	428 clinical isolates	Infant's saliva	Phenotypic testing
				CFA analysis
IV	17 patients	98 clinical isolates	Failed dental implant fixtures (n=33)	Phenotypic testing
				Partial sequencing of the 16S rRNA gene
V	---	4 clinical isolates	Failed dental implant fixtures (n=3) Neck abscess (n=1)	----
unpublished	---	47 clinical isolates	Failed dental implant fixtures (n=33)	

In Study I, to assess the uniformity of reactivity by different test systems, the reference strains were additionally tested with the API ZYM kit (bioMerieux, Marcy l'Etoile, France), and tests based on 4-methyl-umbelliferyl-linked substrates (4-MU; SIGMA, St. Louis, USA). Inocula for testing enzyme activities were from 3-4 days' growth on Brucella agar plates and adjusted to cell turbidity of McFarland #5-6 in sterile water for API ZYM. To evaluate the applicability of different commercial kits for identification of *Actinomyces* species, RapID ANA II system (Remel, Lenexa, Kansas, USA), the Rapid ID 32 A system (bioMerieux, Marcy l'Etoile, France), the Rapid CB Plus system (Remel, Lenexa, Kansas, USA) and BBL Crystal ANR ID system (Becton Dickinson Microbiology System, Cockeysville, MD) were used.

4.2.2 Sequence analysis of the 16S rRNA gene

Bacterial DNA was extracted from pure cultures and the 16S rRNA gene was amplified as described previously by Jalava & Eerola (1999). The DNA sequencing was done using API PRISM DNA 310 Genetic Analyzer (Applied Biosystems). Fragments of the gene about 450 nucleotides long were sequenced. The sequences were determined and compared to the sequences of GenBank (Maidak *et al.* 2001; Benson *et al.* 2004). The sequencing primers have been previously described by Jalava *et al.* (1995).

4.3 Study specimens and microbiological procedures (study III & IV)

Isolates from infants: Altogether 428 potential *Actinomyces* isolates originally detected from both nonselective, aerobically or anaerobically incubated media and from *Actinomyces*-selective CFAT agar were included in further testing. Thirty-nine healthy Caucasian infants from whom saliva samples had been collected on all five scheduled sampling occasions at 2, 6, 12, 18, and 24 months of age (Könönen *et al.* 1999) were included in this study. None of the infants at 2 months, 15 infants at 6 months, and all infants at 12 months had one or more erupted teeth.

Isolates from explanted dental implants: A total of 115 potential *Actinomyces* strains isolated from both nonselective media and CFAT agar were included in further testing including metabolic end product analyses by GLC. The isolates producing major succinic acid were included in identification of *Actinomyces* to species level using the biochemical identification scheme developed in Study I. Thirty-three explanted dental implant fixtures from 17 patients (12 males and 5 females; mean age 54 years, range 25-74 years) with altogether 20 episodes of suspected infectious implant disintegration were included in this study. The fixtures were surgically removed under local anaesthesia and the whole fixture was vortexed to remove attached bacterial cells for further microbiological procedures.

4.4 Titanium plates (Study V)

4.4.1 Handling procedures of the plates

Sixteen commercial pure titanium plates (15 mm x 10 mm x 1 mm) were prepared for the study using a standard procedure for titanium applied in dental laboratories. Briefly, eight of the plates were polished (smooth surface) and the other eight of the plates were sandblasted (rough surface). Four rough and four smooth discs were further coated with bovine-serum-albumin (BSA). Bacterial suspensions corresponding to McFarland 0.5 turbidity were made in sterile water by harvesting three days' growth on anaerobically-incubated Brucella agar plates. The suspensions were mixed with a vortex mixer to avoid cell clumps and viable colony counts were determined to verify the quantity of bacterial cells present in the suspensions. Titanium plates were immersed in 4 ml of cell suspension containing approximately 1.5×10^7 cells/ml and incubated anaerobically at 35-37°C for 22 h. After incubation, the samples were carefully rinsed twice with PBS (0.2 M, pH 7.4) to remove loosely attached cells.
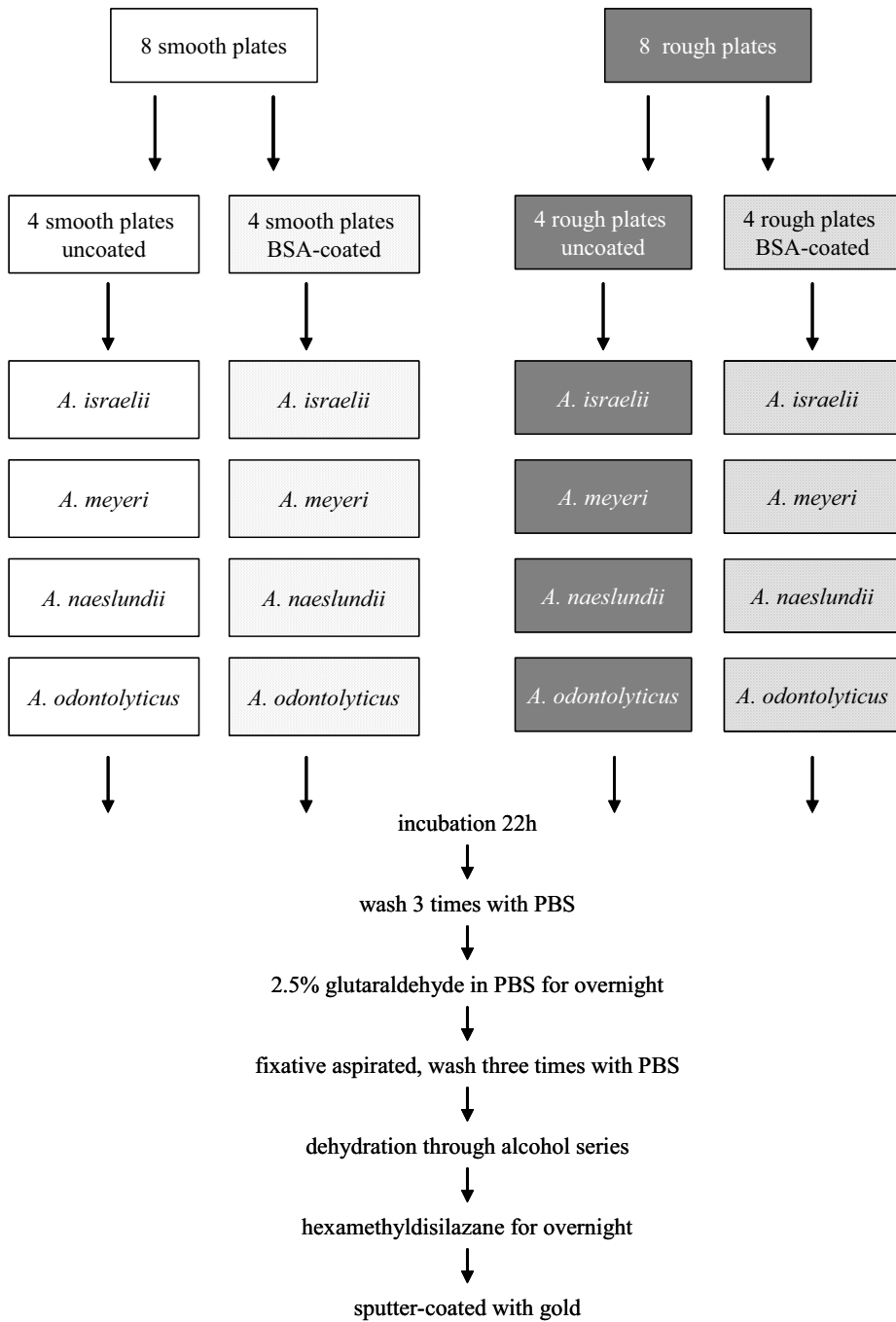
4.4.2 Scanning electron microscopy

Representative titanium plates each colonized with an indicated *Actinomyces* species were prepared for SEM according to a standard procedure presented in Figure 1. The samples were examined using a high-resolution field emission scanning electron microscope (FESEM-6335F, JEOL, Japan). At least six randomly chosen individual fields of each titanium plate were observed under FESEM, and electron micrographs were taken at magnification of x1,000 (sixteen micrographs) and for the purpose of quantification of attached *Actinomyces* cells x2,500 (5 x 16 micrographs). The bacterial cells present on each field were counted by marking each cell on computer display with Photoshop® and the mean number of cells in each of the five fields was determined and expressed as cell numbers/field.

4.5 Statistical methods (Study V)

Statistical analysis was performed with two-way analysis of variance. The ability of different *Actinomyces* species to adhere was compared to analyze the effect of the surface coating (BSA-coated or non-coated) and the surface roughness (rough or smooth) in these analyses. The level of statistical significance was considered to be <0.05.

Figure 1.

8 smooth plates

8 rough plates

4 smooth plates uncoated

4 smooth plates BSA-coated

4 rough plates uncoated

4 rough plates BSA-coated

A. israelii

A. meyeri

A. naeslundii

A. odontolyticus

incubation 22h

wash 3 times with PBS

2.5% glutaraldehyde in PBS for overnight

fixative aspirated, wash three times with PBS

dehydration through alcohol series

hexamethyldisilazane for overnight

sputter-coated with gold

5 RESULTS AND DISCUSSION

5.1 Biochemical identification (Study I & II)

5.1.1 Conventional testing (Study I)

Classically, *Actinomyces* species have been described as branching gram-positive rods. However, many of them are seldom branching, but rather forming a heterogenous group of long or even quite short, curved or straight rods. *Actinomyces* is suspectible to special potency antimicrobial disks vancomycin and kanamycin and resistant to colistin. The genus can be reliably differentiated from other gram-positive non-spore-forming rods by their metabolic end products determined by GLC. Without the demonstration of succinic acid as their metabolic end product, the separation of *Actinomyces, Arcanobacterium,* and *Actinobaculum* from each other can be very difficult. However, the facilities for the measurement of metabolic end products in clinical microbiology laboratories are not often available.

5.1.2 Summary of the identification table (Study I)

Key reactions of different *Actinomyces* species in biochemical testing are depicted in Table 4. Deviating from the current literature, not only *A. odontolyticus* but also three other *Actinomyces* species, *A. graevenitzii, A. radicidentis,* and *A. urogenitalis,* proved to produce pigment on RLB agar after incubation for 5 days. Colonies of *A. odontolyticus* produced brown or purple red, those of *A. graevenitzii* dark, almost black, those of *A. radicidentis* brown, and those of *A. urogenitalis* reddish pigmentation. However, coinciding with the original description of *A. graevenitzii* (Ramos *et al.* 1997), *A. graevenitzii* colonies grown on Brucella agar were non-pigmented, crumpled colonies. Colonies of the type strain of *A. radicidentis* were brownish, whereas those of *A. urogenitalis* were pinkish beige on Brucella agar after 5 days of incubation resembling colonies of *A. odontolyticus* (pinkish, "old rosa"). It is noteworthy that many other *Actinomyces* strains may exhibit some brownish color after prolonged incubation for 6-11 days (Brander and Jousimies-Somer 1992) or when an anaerobically grown culture is allowed to stand aerobically at room temperature (Peloux *et al.* 1985). These are not usually regarded as real pigment production but rather a result of medium decomposition. Thus, growth media and incubation methods can have an impact on pigment production. For example, in a previous report (Kaetzke *et al.* 2003) where *A. odontolyticus* was grown on brain heart blood agar, pigmented colonies appeared only after 48 h, when they usually require 5 days of incubation to develop on Brucella agar.

In a positive catalase test, the enzyme catalyses the breakdown of H_2O_2 with the release of free oxygen. Catalase production has previously been considered a key

characteristic for *A. viscosus* only. However, two additional catalase-producing *Actinomyces* species, *A. neuii* (Funke *et al.* 1994) and *A. radicidentis* (Collins *et al.* 2000) currently exist in the genus. Pigment production and CAMP reaction are useful tests to confirm the separation of catalase-positive *Actinomyces* species (*A. radicidentis* produces pigment and *A. neuii* is CAMP positive). *A. neuii* can be further differentiated to subspecies level by nitrate reaction (*A. neuii* ssp. *anitratus* is nitrate negative whereas *A. neuii* ssp. *neuii* is nitrate positive) (Funke *et al.* 1994). In the present study, the type strain of *A. neuii* ssp. *anitratus* proved to be lipase-positive and *A. neuii* ssp. *neuii* lipase-negative, another distinct characteristic that may be used to separate these two subspecies.

The developed identification scheme relies upon traditional phenotypic identification utilizing biochemical tests, including enzymic reactions and sugar fermentations. The reactions presented in the scheme are obtained from tests of both reference and clinical strains but also from literature, including the descriptions of individual *Actinomyces* species. The wide test pattern we developed has numerous strengths and it takes into account the discrepancies between different phenotypic test systems. Alternative molecular biology techniques are often not available in routine clinical microbiology laboratories. For this reason, it would be important to have phenotypic methods at hand enabling reliable discrimination of *Actinomyces* at species level. Key reactions achieved with conventional identification can be invaluable when confirming suggested identification obtained by partial 16S rRNA gene sequencing. One advantage of culture methods is that they enable the detection of new bacterial properties, e.g., pigment production, as was shown for *A. graevenitzii* (Study III).

However, phenotypic testing of bacterial isolates is time-consuming and needs lots of facilities in clinical microbiology laboratories. As stated in Study I, it would be of the utmost importance to carefully compile the identification tables in publications and manuals. The method(s) by which the reactions have been obtained should be noted to help the interpretation of test results and allow the individual tests to be reproducible. Variations between the test results in the present study and previously published reports were mainly due to differences in enzymic activities utilized by different systems. The discrepancies may also be explained by different substrate specificities and buffering conditions in these test systems. It can be speculated whether the growth conditions for preparation of the inoculum are conducive or inhibitory for the synthesis of a specific enzyme (Bascomb and Manafi 1998). In addition, most of the schemes and algoritms used for identification are tested only for reference strains with predictable biochemical properties under optimal growth conditions (Petti *et al.* 2005). Despite the limitations, phenotypic identification seems to be a considerably powerful method for *Actinomyces*.

Table 4. Key reactions of different *Actinomyces* spp. in biochemical testing

Species	Pigmentation, CAMP, catalase	Enzymic reactions	Sugar fermentations
A. europaeus	pigm-, cat-	β-NAG-, l-ara-	raf-, rhamn-, suc-, tre-
A. funkei		α-fuc+, l-ara+	
A. georgiae	pigm-, cat-	urea-, esc+, β-NAG-, l-ara-	raf-, rhamn+, suc+, tre+
A. gerencseriae	pigm-, cat-	urea-, esc+, β-NAG-, l-ara-	raf+, rhamn-, suc+
A. graevenitzii	adherent colonies pigm+ on RLB agar	β-NAG+	
A. israelii	(molar tooth colony)	urea-, l-ara+	arabinose+
A. meyeri		esc-, β-gal+, β-NAG+	
A. naeslundii		urea+, esc+	
A. neuii ssp. *neuii*	cat+, nitr+, CAMP+		
A. neuii ssp. *anitratus*	cat+, nitr-, CAMP+		
A. odontolyticus	pigm+	α-fuc+, β-NAG-	
A. radicidentis	pigm+ on RLB agar, cat+		
A. radingae	cat-, CAMP+	esc+, α-fuc+, β-NAG+	
A. turicensis		β-NAG-, β-gal-, esc-	
A. urogenitalis	pigm+ on RLB agar	β-NAG+, esc+	
A. viscosus	cat+, CAMP-		

5.1.3 Commercial identification systems (Study II)

When compared to conventional biochemical identification, our data indicate a relatively poor applicability of the commercially available test kits for reliable identification within the genus *Actinomyces*. When commercial kits were used in identification of the *Actinomyces* strains, approximately half of them were identified to the species level: 40% by RapID ANA II, 58% by Rapid ID 32 A, 26% by RapID CB Plus, and 65% by BBL Crystal. In previous studies, variable percentages have been reported: the RapID ANA II system identified 24% (Miller *et al.* 1995) and 85% (Brander and Jousimies-Somer 1992), RapID CB Plus 52% (74% with extra tests) (Hudspeth *et al.* 1998), and BBL Crystal 97% (Cavallaro *et al.* 1997) of the *Actinomyces* species tested. According to our experience, the main inconsistencies were encountered among glycosidases (α-glucosidase, β-glucosidase), sugar fermentations (glucose, maltose, ribose, sucrose), aminopeptidases (pyrrolidonyl aminopeptidase), and urease. Conventional methods, including the use of PRAS carbohydrate fermentation tests and individual diagnostic tablets for enzymic reactions, seemed to be more accurate, but much more time-consuming than the commercial kits. The commercial identification kits examined in this study clearly need some corrections and additional tests to their databases, and these kits should be developed in order to be able to identify the newly described *Actinomyces* species as well. According to the present results, commercial kits did not seem to offer a reliable method for identification of *Actinomyces*. However, BBL Crystal and RapID 32 A may be helpful for rapid preliminary identification of *Actinomyces* and closely related species in clinical microbiology laboratories. Despite the poor percentages of identification, our results are in accordance with those of Clarridge and Zhang (2002) showing that, although the biochemical identifications with commercial kits frequently indicate an inaccurate species name and poor identification, many of the different genogroups show distinctive and reproducible biochemical profiles which can be valuable in their detections.

5.2 16S rRNA sequencing (unpublished results)

Genotypic methods based on partial 16S rRNA sequence analysis of *Actinomyces* revealed to be a useful method for genus level identification, and it also improved the phenotypic identification to species level. Phenotypic identification of the isolates correlated with that obtained by partial 16S rRNA sequencing (similarity index ≥97%) for 44/47 (94%) isolates at the genus level and for 30/47 (64%) isolates at the species level. For most isolates interpreted as identified to genus level by 16S rRNA sequencing, a different *Actinomyces* species level identification was suggested by the database as compared to phenotypic identification. In addition, phenotypic identification of 7 isolates correlated with sequence identification but with lower similarity index (94-96%). A correlation of the phenotypic and sequence-based identification at species level was in most cases obtained with *A. israelii* and *A. naeslundii.* Probably due to insufficient database, many *A. odontolyticu*s isolates were identified as *A. meyeri* as the first choice. When partial sequencing was used in identification of *Actinomyces*, additional biochemical testing was needed to confirm the identification at species level.

Public databases, such as the GenBank, are used to analyze sequencing results for identification. However, this bring us many problems: 1) quality of entries in GenBank is compromised by sequencing errors, incomplete sequences, and insufficient strain characterization (Drancourt *et al.* 2000; Patel 2001; Boudewijns *et al.* 2006), 2) there are no universally accepted data on how to interpret sequence-based results, 3) the length of sequenced part of the gene (400-500 bp) includes some restrictions. Taking these aspects into account, it would be reasonable to use partial 16S rRNA sequencing primarily as an adjunct to phenotypic key methods and the results of partial sequencing should be interpreted together with phenotypic results.

5.3 Oral *Actinomyces* in infants (Study III)

Already at the age of 2 months, *Actinomyces* species were detected in the edentulous oral cavity. The frequency and number of species increased in proportion with age: 30% of the 39 infants at 2 months but 97% at 2 years of age were positive for salivary *Actinomyces*. As seen in Fig. 2, *A. odontolyticus* was the most predominant *Actinomyces* colonizer on all five sampling occasions. Similar frequencies were found in 19 predentate and 13 dentate infants at 6 months (59% and 41%, respectively). This is contradictory to the study of Cole *et al*. (1998) who failed to detect any *Actinomyces* species prior to four months after tooth eruption and no *A. odontolyticus* during the study period (mucosal surface samples from infants between the age of 2 - 24 months). On the other hand, Ellen (1976) detected catalase-negative *Actinomyces* in 40% of predentate infants, and due to limited identification properties at that time, these isolates may have been *A. odontolyticus*. The results of the present study suggest that tooth surfaces or gingival crevices are not prerequisites for colonization of *A. odontolyticus,* but rather mucosal surfaces which serve as the initial colonization site and reservoir for other oral sites (Gibbons 1989; Frisken *et al.* 1990; McClellan *et al.* 1996). Intrestingly, in the study by Könönen *et al.* (2003), *Actinomyces* species were only occasionally present in infants' nasopharynges during health, but were dominating species frequently isolated from nasopharyngeal aspirates during acute otitis media episodes. Whether these species colonize the nasopharynx due to environmental changes during infection or whether they have a role in the pathogenesis of acute otitis media, is not yet known. *Actinomyces* species are also known to colonize tonsillar crypts in adulthood (Brook and Foote 1997; Toh *et al.* 2006), and they may also colonize the mucosal surfaces of tonsils in infants soon after birth (Cole *et al.* 2004). Interestingly, among patients with peritonsillar and retropharyngeal abscesses, the most common *Actinomyces* species has been *A. odontolyticus* (Civen *et al.* 1993; Jousimies-Somer *et al.* 1993), thus emphasing its colonizing role at mucosal surfaces.

Figure 2. Prevalence rates of oral *Actinomyces* species in 39 children between the age of 2-24 months
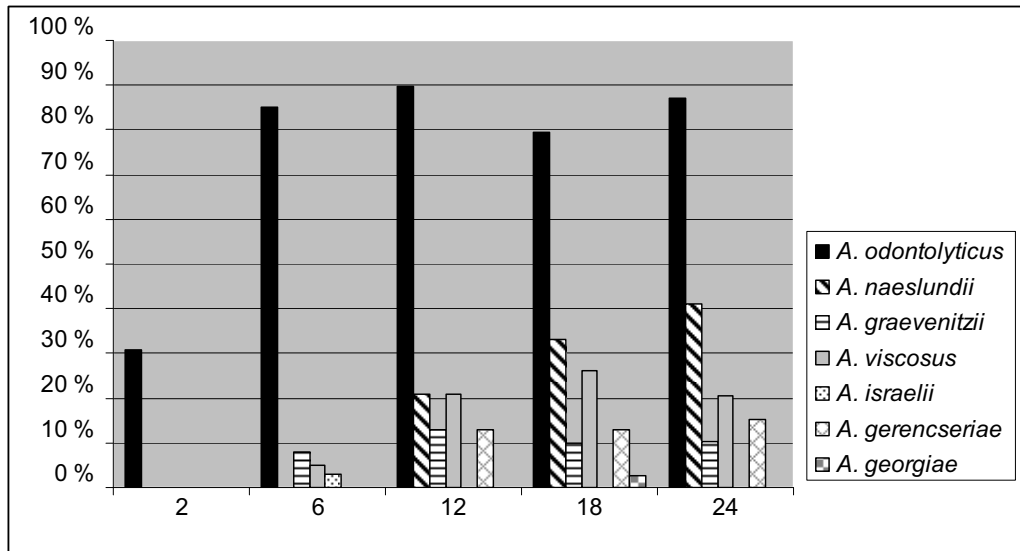
First recoveries of *A. viscosus* were from 6-month-old infants and those of *A. naeslundii* from 12-month-old infants. However, the proportional distribution of *A. naeslundii* was higher than that of *A. viscosus* during the second year of life. Both infants positive for *A. viscosus* at 6 months had at least one erupted tooth. This coincides with the previous observation by Ellen (1976) that the colonization of *A. viscosus* is delayed until teeth have erupted. Tooth surface with salivary pellicle of enamel surface seems to be an ideal environment for the initial adhesion of *A. naeslundii* and *A. viscosus*. In the study by Mager *et al.* (2003), *A. odontolyticus* was found in significantly higher proportions in saliva and specific parts of the tongue compared to other sites, such as attached gingiva in healthy adults. On the other hand, the same study demonstrated that *A. naeslundii* and *A. viscosus* colonize teeth at far greater proportions than soft tissues.

As described in the literature, receptor specificities of bacterial cell-surface adhesins might determine the abilities of *A. odontolyticus, A. naeslundii,* and *A. viscosus* to colonize different oral sites correlating with the binding specificities of each species to beta-linked galactosamine and acidic proline-rich protein structures (Strömberg and Boren 1992; Hallberg *et al.* 1998). In addition to initial adhesion to oral surfaces, specific associations between *Actinomyces* species and subsequently colonizing bacteria have been identified suggesting further contribution to biofilm development

(Palmer *et al.* 2003; Foster and Kolenbrander 2004; Jakubovics *et al.* 2005). Könönen *et al.* (1994; 1999) indicated that anaerobic bacterial species have a different suspectible time for their colonization, probably due to interrelationships in the bacterial succession.

The use of our comprehensive identification test arrays enabled characterization of early colonizing *Actinomyces* species in infants' mouths *A. odontolyticus* being the most prominent colonizer followed by *A. naeslundii* and *A. viscosus*. The developed scheme also resulted in novel observations of *A. graevenitzii, A. gerencseriae,* and *A. georgiae* isolated from the oral cavity in infancy. *A. graevenitzii* was isolated from infants' saliva from 6 months of age onwards. The four previous *A. graevenitzii* strains in the original description (Ramos *et al.* 1997) were mainly isolated from respiratory tract secretions, and most probably were of oral origin.

5.4 *Actinomyces* findings in failed dental implant fixtures (Study IV)

Actinomyces was nearly ubiquitous finding in failed implants being present in 94% of the 33 explanted fixtures. This was not surprising, since *Actinomyces* species are considered to be initial colonizers on tooth surface particularly related to the accumulation phase of plaque development (Liljemark *et al.* 1993). Bacterial attachment and plaque accumulation is also considered as a key factor in the pathogenesis of biomaterial-associated infections leading to possible failure of implants (Gristina 1987). Several reports on infections connected with intrauterine contraceptive devices and prosthetic hip joints with *Actinomyces* have been published (Yoonessi *et al.* 1985; Wust *et al.* 2000; Zaman *et al.* 2002; Elsayed *et al.* 2006). Besides *Actinomyces*, other common findings in the present study were *Fusobacterium* species, pigmented *Prevotella* and anginosus group streptococci, being isolated from 85%, 76% and 73% of the implants, respectively. In the study of Gerber *et al.* (2006), the most prevalent species in plaque on implant surfaces (curette samples from implant surfaces) consisted of streptococci, *Veillonella*, *Capnocytophaga*, *Fusobacterium*, and *Neisseria*, as well as periodontal pathogens *P. gingivalis, A. actinomycetemcomitans,* and *Actinomyces.* Little is known about the mechanisms of bacterial interactions on oral implant surfaces. As *Actinomyces* are known to co-aggregate with most of the above mentioned species during plaque formation (Kolenbrander and Phucas 1984; Kolenbrander and Andersen 1986; Kolenbrander 1988), it can be speculated that same kinds of partnerships are also valid in the environment around implants. Implants are known to frequently become colonized by opportunistic bacteria that form biofilms on these implant surfaces (Costerton *et al.* 1999; Costerton *et al.* 2005).

Classically, the microbiota of failed implants has been considered to be very similar to that of periodontitis, and many studies have focused only to detect periodontal pathogens from these implant sites. Numerous studies have reported microbial findings in failing dental implants (Mombelli *et al.* 1988; Becker *et al.* 1990; Leonhardt *et al.* 1993; Keller *et al.* 1998; Leonhardt *et al.* 1999; Grossner-Schreiber *et al.* 2001;

Leonhardt *et al.* 2002; Buchmann *et al.* 2003; Gerber *et al.* 2006). However, the interpretation and comparison of these bacteriological data is very difficult or almost impossible because various methods have been used for bacteriological analyses. Samples in reported studies have been collected from the depth of peri-implant sites by using paper points or curettes. In addition, gingival crevicular fluid (GCF) samples from implants have been reported (Gerber *et al.* 2006). However, according to our unpublished observations, the implant fixture seems to be 100-1000 times more effective as a sampling material compared to scoop samples in terms of qualitative and quantitative yields of cultivable bacterial groups and species. In the present study, each fixture was explanted and then used for bacteriological investigation as a whole, thus enabling us to investigate the true bacterial attachment to the implant surface. Also, the timeframe between the insertion of an implant and infection can affect the detection of bacteria: samples have been taken between 0 months to 10 years after implant insertion (Mombelli *et al.* 1988; Hultin *et al.* 2000). Factors, such as time of implant loading and the status of remaining dentition, seem to reflect strongly the composition of implant microbial complexes.

In the present study, *A. odontolyticus* was by far the most prominent and frequent *Actinomyces* species found, the frequency of detection being as high as 79% in the examined 33 failed implant fixtures. This is in line with the finding of *A. odontolyticus* as the predominant *Actinomyces* species in early plaque formation on tooth surfaces (Liljemark *et al.* 1993) and on oral mucosal surfaces of infants (Study III). *A. odontolyticus* may also play a significant role in the early stages of biofilm formation on dental implant surfaces. Interestingly, a significant increase of *A. odontolyticus* cells in subgingival plaque was observed in edentulous patients with failing implants (periimplant pocket depth of ≥6 mm and pus formation) compared to those with successful implants (Mombelli *et al.* 1988). Also in edentulous patients, the presence *A. odontolyticus* correlated with probing depth in 18 implants (Mombelli and Mericske-Stern 1990). In these cases *A. odontolyticus* must have been of mucosal surface origin confirming our finding that tooth surface is not a prerequisite for *A. odontolyticus* (Study III). Further, in a review on infections caused by *A. odontolyticus* (Peloux *et al.* 1985), it was presented that other mucous membranes than those in the mouth carry this specific organism. Lower detection rates for *A. odontolyticus, A. naeslundii,* and *A. viscosus* in peri-implant sites were reported by Rutar *et al*. (2001) than the corresponding rates presented in our study (36% vs.79%, 19% vs. 30% and 17% vs. 30% respectively). Their lower rates may be explained by a different sampling method, *i.e.*, paperpoint sampling, which may not be optimal for detecting attached cells. Also, an interesting finding was observed in the study by Keller *et al.* (1998), who detected *A. naeslundii* from peri-implant microbiota only from pockets ≥ 4mm. Gerber *et al*. (2006) compared bacterial plaque samples collected from titanium implant and tooth surfaces. *A. naeslundii* was detected with almost similar frequencies on both surfaces, whereas detection rates of *A. odontolyticus* and *A. israelii* were higher on implant than tooth surfaces. Bacteria that cause implant-associated infections live in well-developed biofilms (Costerton *et al.* 1999). These infections are considered multi-species rather than caused by a single species (e.g., a periodontal pathogens). By reducing the number of initially adhering species, such as *Actinomyces* and streptococci, on these surfaces would make it possible to minimize early plaque formation and subsequent inflammation in implant-surrounding soft tissues.

5.5 Adhesion of *Actinomyces* to titanium plates (Study V)

All tested *Actinomyces* species adhered to all differently processed Ti surfaces. However, both the degree of surface roughness and albumin coating of titanium revealed significant differences (p<0.05) between different *Actinomyces* species regarding their adhesion to titanium. The most prominent colonizer on various titanium surfaces was *A. meyeri*. These findings are in line with previous observations that different oral bacteria attach to surfaces in a selective manner (Gibbons and van Houte 1975; Mabboux *et al.* 2004). As suggested by Ellen (1976) and Gibbons (1989), the members of the genus *Actinomyces* may differ in their selectivity in adhering onto oral surfaces. According to present results, this also seems to be the case concerning titanium surfaces.

Initial bacterial adhesion is affected by many factors, including bacterial characteristics, environmental factors (e.g. the presence of serum proteins), and material properties of the target surface (Brecx *et al.* 1983; An and Friedman 1998). Roughening of the surface increases the area available for adhesion, creating pits and grooves, therefore providing favorable sites for colonization (Nakazato *et al.* 1989; Bollen *et al.* 1996). However, it is still unclear whether all early-colonizing species prefer rough and irregular areas for initial adhesion and how the adhesion mechanisms change under different conditions (e.g., subgingivally located titanium surface of an implant). In the present study, an uncoated, smooth titanium surface promoted the adhesion of *A. israelii* and *A. meyeri,* whereas in the presence of BSA *A. israelii* and *A. meyeri* showed the highest numbers of adhered cells on rough surface*. A. naeslundii* preferred the smooth surfaces, with the highest numbers of adhered cells being present on the BSA-coated surface. *A. odontolyticus* preferred the rough surfaces*.*

The presence or absence of BSA-coating had a significant effect on the adhesion of *A. naeslundii, A. meyeri,* and *A. israelii*. BSA had a promoting effect on rough surfaces for *A. israelii* and *A. meyeri,* and on both smooth and rough surfaces for *A. naeslundii.* This illustrates the diversity of adhesion mechanisms of these species to biomaterials*.* The interaction effects were significant with *A. meyeri* and *A. israelii:* the effect of BSA was dependent on the roughness of the target surface. These results showing a stimulating effect of albumin coating were unexpected because human serum albumin has proved to be an effective method in preventing bacterial adhesion on implant materials (An *et al.* 1996; An *et al.* 1997). Albumin is known to be an acidic protein and capable of reducing the substrate surface hydrophobicity, showing strong inhibitory effects on bacterial adhesion (Reynolds and Wong 1983; Brokke *et al.* 1991; An and Friedman 1998). However, according to our results, the effects of BSA seemed to depend on bacterial species and strains. Coating with BSA reduced the numbers of *A. odontolyticus* cells found on both smooth and rough surfaces. Our studies suggest that *A. odontolyticus* has unique binding sites to adhere on titanium. The species is known to exhibit a genetically related, but functionally distinct adhesin, which is structurally different from fimbriae (Hallberg *et al.* 1998) .

Initial adhesion of four different *Actinomyces* species to titanium surfaces was the main focus of the present study. Initial adhesion of early colonizing species is known to be the preliminary stage in the further development of multi-bacterial biofilms where coaggregation, co-adhesion, and competition between bacterial species largely influence plaque formation *in vivo*. Therefore, an *in vitro* experiment, such as the present one, has it limitations when studying adhesion properties. Exposure of only one *Actinomyces* species to titanium surface without other species contributing to biofilm formation differs from a true clinical situation. However, studies on initially adhering organisms are of great importance, because the formation of plaque within the first hours occurs with these adhered *Actinomyces* species (Li *et al.* 2004). Further, a surface capable of stimulating or inhibiting the initial adhesion of early species, such as *Actinomyces* (which can ultimately influence the succeeding stages of plaque biofilm formation via their coadhesion properties), may be crucial to developing biofilm on biomaterial surfaces.

6 KEY FINDINGS AND CONCLUSIONS

Study I, II, unpublished

The developed phenotypic scheme with advanced diagnostic methods will be valuable asset in clinical laboratories for species level identification of the genus *Actinomyces*. Although the identification of these gram-positive rods may pose major problems, it is important to characterize *Actinomyces* to species level to increase the knowledge of their natural habitats and clinical associations.

When compared to conventional phenotypic methods, considerable inconsistency existed in the ability of commercial kits to identify *Actinomyces* species. None of the examined commercial test systems was able to identify all *Actinomyces* correctly. Lack of updated information in databases interfered with precise identification rendering the methods unreliable for species level identification.

Partial 16S rRNA sequence analysis increased the accuracy of identification of *Actinomyces* to species level. However, due to problems in interpretation and quality of current databases, this method may only be reliably used as an adjunct to phenotypic methods.

Study III

Actinomyces species were found in edentulous infants at 2 months of age, *A. odontolyticus* being the most prominent species. The frequencies of *A. naeslundii*, *A. viscosus* and *A. gerencseriae* increased with age after the second half of the first year. This suggests that *A. odontolyticus* is an inhabitant of mucosal surfaces whereas tooth surfaces are prerequisites for other oral *Actinomyces* species. Different *Actinomyces* species seem to find unique niches of colonization at a time convenient for them.

Study IV

Actinomyces species formed a major part of bacterial species isolated from failed dental implant fixtures. *A. odontolyticus* was the most prominent finding. This may suggest that *A. odontolyticus* is a primary colonizer on failed implant surfaces as a part of multispecies bacterial community.

Study V

Distinct *Actinomyces* species differed significantly in their adherence onto various titanium surfaces. Surface roughness and albumin coating had significant effects on the adhesion. This may confirm previous results that different *Actinomyces* species adhere in a selective manner onto oral surfaces.

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