VIRULENCE-ASSOCIATED CHARACTERISTICS OF

*ACTINOBACILLUS ACTINOMYCETEMCOMITANS*,

AN ORAL AND NONORAL PATHOGEN

Susanna Paju

Helsinki 2000
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AN ORAL AND NONORAL PATHOGEN

Susanna Paju

Academic dissertation

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the main auditorium of the Institute of Dentistry, Mannerheimintie 172, Helsinki, on 25 February, 2000, at 12 noon.

ISBN 951-45-9129-1 (PDF version)
Helsingin yliopiston verkkojulkaisut
Helsinki 2000
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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications which will be referred to in the text by their Roman numerals:


In addition, some unpublished data are presented.
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AP-PCR</td>
<td>arbitrarily primed polymerase chain reaction</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>CCUG</td>
<td>Culture Collection at the University of Gothenburg</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DnaK</td>
<td>70 kDa heat shock protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>GroEL</td>
<td>60 kDa heat shock protein</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IDH</td>
<td>Institute of Dentistry, University of Helsinki</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LJP</td>
<td>localized juvenile periodontitis</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC at which 50% of isolates are inhibited</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC at which 90% of isolates are inhibited</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PLE cells</td>
<td>periodontal ligament epithelial cells</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIgA</td>
<td>secretory immunoglobulin A</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
INTRODUCTION

Bacterial virulence defines the strength of the pathogenic potential of a microorganism. Several bacterial virulence factors and mechanisms contribute to this potential to cause disease. *Actinobacillus actinomycetemcomitans*, a pathogen not only in periodontal but also in some nonoral infections, possesses several virulence determinants which contribute to its ability to colonize the oral cavity, persist in the periodontal pocket, resist and evade host defenses, cause destruction to soft and hard tooth-supporting tissues, and interfere with host tissue repair after infection (Fives-Taylor et al. 1999).

*A. actinomycetemcomitans* is one of the most thoroughly studied periodontal bacteria. Previous studies on *A. actinomycetemcomitans* have been performed mostly on oral serotypeable *A. actinomycetemcomitans* strains. More information is needed on nonserotypeable *A. actinomycetemcomitans* strains and *A. actinomycetemcomitans* strains from nonoral infections, since they may exert virulence different from that of serotypeable and/or oral *A. actinomycetemcomitans* strains. Some virulence factors of a few *A. actinomycetemcomitans* laboratory strains have been closely studied. New data are required as to the expression capacity of specific virulence factors in clinical *A. actinomycetemcomitans* strains and the effects of this expression on the host. We would then better understand the pathogenic potential and mechanisms exerted by this organism and understand its contribution to periodontal destruction.

The hypothesis of the present studies was that differences exist in the virulence characteristics among *A. actinomycetemcomitans* clones. The aim was to find *A. actinomycetemcomitans* strains with elevated virulence by studying the reasons for the nonserotypeability of *A. actinomycetemcomitans* strains, by phenotypically and genotypically characterizing nonoral *A. actinomycetemcomitans* strains, and by studying the heat-response, a probable virulence factor, of *A. actinomycetemcomitans* and its effects on periodontal epithelial cells.
REVIEW OF THE LITERATURE

Periodontal diseases

Periodontal diseases are inflammatory changes in the periodontium caused by bacterial infection. Gingivitis leads to bleeding, swelling, and redness of the marginal gingiva. Periodontitis leads to destruction of the tooth-supporting tissues, both connective tissue and bone, and eventually to tooth loss. The various forms of periodontal diseases differ from each other in etiology, natural history, and response to therapy, but exhibit similar clinical and histopathological events (Page & Schroeder 1977).

Pathogenesis

The general principles in the pathogenesis of periodontitis established by Page & Schroeder (1976) are still currently accepted, and continuing research since then has added detailed information to elucidate the entire picture of periodontal pathogenesis (American Academy of Periodontology 1992, World Workshop in Periodontics 1996, Page & Kornman 1997). The pathogenesis of the periodontal lesion occurs in initial, early, established, and advanced stages, of which the first three are characterized as gingivitis and the last as periodontitis (Page & Schroeder 1976). In initial and early stages, accumulation of dental plaque in the gingival sulcus enhances the leukocyte and neutrophil migration to the junctional epithelium and underlying connective tissue, results in loss of collagen and fibroblast alteration in the marginal gingiva, and induces the proliferation of basal cells of the junctional epithelium (Page & Schroeder 1977, Schroeder 1996). Later, in the established lesion, plasma cells predominate, the connective tissue loss continues, and the junctional epithelium migrates apically starting conversion to pocket epithelium (Page & Schroeder 1977, Zappa 1995). Destruction of alveolar bone and connective tissue in the gingiva and periodontal ligament, periodontal pocket formation, and several inflammatory reactions take place in the advanced periodontal lesion (Page & Schroeder 1977). Bacteria are etiological agents, but they are not alone responsible for the tissue destruction in periodontitis. Most of the periodontal tissue destruction is due to indirect activity of bacteria: activation of protective and defensive host mechanisms, such as enzymes and cytokines, can lead to destruction of the host tissues.
(Kornman et al. 1997). In the initiation and progression of periodontitis the susceptibility of the host to disease is not yet completely understood. Genetic and environmental factors, including compromised immunity, smoking, and hormonal factors, are suggested to be risk factors for periodontal diseases (American Academy of Periodontology 1992, World Workshop in Periodontics 1996, Page et al. 1997).

**Bacteriology of periodontal infections**


**Characteristics of Actinobacillus actinomycetemcomitans**

*A. actinomycetemcomitans* was first described in 1912 (Klinger 1912) as *Bacterium actinomycetem comitans*, "a bacterium associated with actinomyces."

**General characteristics**

*A. actinomycetemcomitans* is a gram-negative nonmotile coccobacillus. The organism is facultatively anaerobic and capnophilic; it grows well in 5% CO₂ in air or anaerobically, and growth into well-developed colonies takes 48 to 72 h (Slots 1982a). A.
actinomycetemcomitans does not require X (hemin) or V (nicotinamide adenine dinucleotide) factors for growth (Slots 1982a) and grows in the absence of serum or blood (Tsai et al. 1979). Biochemically, A. actinomycetemcomitans is nonhemolytic, produces catalase, does not produce indole, reduces nitrate, and is fermentative (Hammond & Stevens 1982, Slots 1982a). On solid growth media, fresh A. actinomycetemcomitans isolates adhere to agar and form circular colonies, 0.5 to 1 mm in diameter, with slightly irregular edges (Slots 1982a). These phenotypically rough colonies are translucent, with an internal star-shaped morphology (Slots 1982a). Fimbriae on the cell-surfaces make rough A. actinomycetemcomitans colonies adherent to agar (Scannapieco et al. 1987, Rosan et al. 1988). After repeated subcultures, the internal structure of the bacterial colonies may be lost, forming smooth colony types with more opaque colonies, as represented by A. actinomycetemcomitans strains from the American Type Culture Collection (Slots 1982a). A conversion has been observed: from a fimbriated rough phenotype to non-fimbriated smooth phenotype with decreased ability to adhere (Slots 1982a, Rosan et al. 1988, Inouye et al. 1990, Haase et al. 1999).

A. actinomycetemcomitans in the oral cavity and as a periodontal pathogen

The oral cavity is the ecological niche for A. actinomycetemcomitans. Approximately 90% of patients with localized juvenile periodontitis (LJP) and 50% of adult periodontitis patients harbor A. actinomycetemcomitans in the oral cavity (Slots et al. 1980a), and this organism may be isolated from some periodontally healthy subjects as well (Slots et al. 1980a, Asikainen et al. 1986). However, A. actinomycetemcomitans has not been detected in edentulous infants (Könönen et al. 1992). A. actinomycetemcomitans was first isolated and identified to species level in human periodontal lesions in 1979 from a patient with juvenile periodontitis (Baehni et al. 1979). Nearly two decades later, in 1996, it was classified as a periodontal pathogen (World Workshop in Periodontics 1996). This organism fulfills the criteria of an etiological agent of periodontitis (Socransky 1979). A. actinomycetemcomitans is present in high numbers in periodontal lesions, especially in LJP, and absent or in low proportions in healthy periodontium (Slots et al. 1980a, Zambon 1985, Asikainen et al. 1986). The elimination of the organism results in improvement of the clinical periodontal condition (van Winkelhoff et al. 1992). The host with a periodontal infection exhibits elevated serum and salivary antibody levels against this pathogen (Ebersole et al. 1982). A. actinomycetemcomitans possesses virulence factors associated with the pathogenesis and the progression of the disease (Baehni et al. 1979, Kiley & Holt 1980, Shenker et al. 1982, Wilson & Henderson 1995).
Additionally, animal models link the organism to periodontal tissue destruction (Irving et al. 1975).

**A. actinomycetemcomitans in systemic infections**

Besides periodontal infections, *A. actinomycetemcomitans* is occasionally isolated from severe systemic infections (for reviews see Zambon 1985, van Winkelhoff & Slots 1999). It has been detected, either as a causative agent or as a co-pathogen, from native- or prosthetic-valve endocarditis (Pierce et al. 1984, Kristinsson et al. 1988, Kaplan et al. 1989, Verhaaren et al. 1989, Braconier & Söderström 1990, Chen et al. 1991, el Khizzi et al. 1997), pericarditis (Horowitz et al. 1987, Zijlstra et al. 1992), septicemia (van Winkelhoff et al. 1993), pneumonia (Morris & Sewell 1994, Yuan et al. 1992), infectious arthritis (Molina et al. 1994), and abscesses in various body sites, such as brain, submandibular space, or hand (Salman et al. 1986, Kaplan et al. 1989, Zijlstra et al. 1992). The majority of reported nonoral *A. actinomycetemcomitans* infections are case reports, and the information on the microbiological characteristics of nonoral *A. actinomycetemcomitans* strains is limited.

Approximately 0.6% of infective endocarditis cases are estimated to be caused by *A. actinomycetemcomitans* (Das et al. 1997). The true number of nonoral *A. actinomycetemcomitans* infections may be higher, since the organism may be difficult to grow and identify in most laboratories, and therefore may remain unrecognized or be misidentified (Morello 1980, Das et al. 1997). Recently, periodontitis has been associated with chronic coronary heart disease (Mattila et al. 1995, Beck et al. 1996), and *A. actinomycetemcomitans* has been identified in atheromatous plaques in coronary arteries (Zambon et al. 1997). Besides the classic coronary risk factors, periodontal pathogens may play a role in the pathogenesis of coronary heart disease (Mattila et al. 1998).

**Serotype classification of *A. actinomycetemcomitans***

Several techniques, such as immunodiffusion (Zambon et al. 1983, Saarela et al. 1992) and indirect immunofluorescence (Zambon et al. 1983, Asikainen et al. 1991) with polyclonal antibodies, or indirect immunofluorescence with monoclonal antibodies (Gmüür et al. 1993), have been carried out to serologically characterize *A. actinomycetemcomitans*. Up to now, five *A. actinomycetemcomitans* serotypes have been designated (a, b, c, d, and e) (Zambon et al. 1983, Saarela et al. 1992). However, 3% to 8% of *A. actinomycetemcomitans* isolates remain nonserotypeable (Saarela et al. 1992, Gmüür et al. 1993, Poulsen et al. 1994). Most periodontal
patients with *A. actinomycetemcomitans* infections harbor only one serotype (Slots et al. 1982, Zambon et al. 1983, Saarela et al. 1992, Dogan et al. 1999a). Multiple serotypes (or serotypeable and nonserotypeable isolates) are found in less than 10% of the subjects (Saarela et al. 1992, 1999). The serotype distribution of *A. actinomycetemcomitans* in the oral cavities of periodontal patients seems to be heterogeneous: serotype a in 25%, b in 29%, c in 23 to 26%, d in 3 to 4%, e in 6 to 10%, and nonserotypeable isolates in 3 to 5% of subjects (Saarela et al. 1992, Dogan et al. 1999a). The only study on the *A. actinomycetemcomitans* serotype distribution in nonoral infections suggested the predominance of serotype c in 72% of subjects (Zambon et al. 1988). After the study of Zambon and coworkers (1988), two novel *A. actinomycetemcomitans* serotypes, serotypes d and e, were found (Saarela et al. 1992, Gmüür et al. 1993). Studies on serotype distribution of nonoral *A. actinomycetemcomitans* strains which take these two new serotypes into consideration have not been carried out.

**Genotype classification of *A. actinomycetemcomitans***

Molecular typing techniques, including restriction endonuclease analysis (Zambon et al. 1990, van Steenbergen et al. 1994), restriction fragment-length polymorphism (DiRienzo et al. 1990, Slots et al. 1993), the arbitrarily primed polymerase chain reaction (AP-PCR) (Slots et al. 1993, Asikainen et al. 1995), and ribotyping (Alaluusua et al. 1993, van Steenbergen et al. 1994), have been used to genotypically characterize *A. actinomycetemcomitans* isolates. Of these methods, ribotyping has produced the most heterogenic variety of genotypes, but is the most time-consuming and laborious technique. AP-PCR distinguishes approximately 20 *A. actinomycetemcomitans* genotypes, the amount depending on the primers used, and is a rapid and simple technique. *A. actinomycetemcomitans* isolates of a given AP-PCR genotype usually belong to the same serotype (Asikainen et al. 1995), indicating a systematic genetic dissimilarity between serotypes. Additionally, ribotyping (Saarela et al. 1995) and restriction endonuclease analysis (Zambon et al. 1990, van Steenbergen et al. 1994) show the serotype-specificity of certain *A. actinomycetemcomitans* clones.
Background of host-pathogen interaction

Host defense

Against bacterial pathogens each host exhibits non-specific and specific defense mechanisms (Male & Roitt 1998). The non-specific host defense mechanisms, such as intact skin and mucosa, the cleansing effect of body fluids or ciliary action, and phagocytes in tissues and blood, are always present and aimed against all pathogens. The specific host defense by humoral and cellular immunity, including antibodies, activated macrophages, and cytotoxic T cells, is directed towards a specific target and induced after the appearance of the target. The colonization of pathogenic flora is made difficult in the skin, oral cavity, upper respiratory tract, colon, and vagina by the resident commensal microbiota, which are generally non-pathogenic in their natural habitat. However, the presence of commensal bacteria in severe infections is not rare; 63% of infective endocarditis episodes are caused by streptococci (Bayliss et al. 1983), of which oral “viridans streptococci” represent 50% of cases (Knox & Hunter 1991).

When interacting with the host, the pathogen first comes into contact with the epithelial cells lining the mucosal surfaces of the oral cavity or respiratory, intestinal, or genitourinary tracts. Besides functioning as a barrier against exogenous invaders, the epithelial cells sense the microbial infection and provide signals to the host, transmit information between epithelial cells and the cells in the underlying connective tissue, and activate a wide range of inflammatory and immune responses (Kagnoff & Eckmann 1997). The response of intestinal epithelial cells to enteric bacteria depends on the invasion capability of the bacteria (Kagnoff & Eckmann 1997). For instance, Salmonella, Yersinia, Shigella, and Listeria, species that penetrate the epithelium and invade the gut mucosa, induce a rapid expression of inflammatory cytokines in epithelial cells. Clamydia, which invades cervical and intestinal epithelial cells but not the underlying mucosa, initiates a delayed inflammatory response by directly activating the epithelial cells. Many of the epithelial-cell responses evoked by bacteria contribute to epithelial-cell growth, development, and death (Kagnoff & Eckmann 1997, Zychlinsky & Sansonetti 1997).
**Bacterial virulence**

Pathogenicity is defined as the ability of a microbe to produce disease and cause damage in the host (Salyers & Whitt 1994). Virulence determines the strength of the pathogenic potential and defines the relative capacity of a microbe to cause damage in the host and its ability to overcome host defenses (Casadevall & Pirofski 1999). Virulence includes invasiveness, tissue-destructive capacity, and evasion of host responses. These characteristics are individual to each pathogen. Recently, degree of host damage has been suggested as a means of characterizing microbial pathogenicity (Casadevall & Pirofski 1999).

The susceptibility of the host plays a role in infectious diseases. Pathogens that cause disease in some people may be harmless colonizers in others. Approximately 80% of the exposed population may carry *Neisseria meningitidis* in their noses or throats during a meningitis outbreak, but only a small proportion develop the disease (Salyers & Whitt 1994). Additionally, clonal diversity within species may contribute to the outcome of the disease. For instance, *Escherichia coli* strains causing gastrointestinal infections will not cause urinary tract infections or meningitis, because the strains have a different pathogenic potential to cause disease in different host tissues (Salyers & Whitt 1994, Falkow 1997).

The host environment regulates the expression of bacterial virulence genes (Guiney 1997). During the course of an infection, the temperature, pH, osmotic strength, and oxygen and iron availabilities in the growth environment may change dramatically, requiring bacterial adaptation to the new environment (Guiney 1997, Falkow 1997). Temperature-sensitive gene expression, needed for instance in the invasion of colonic epithelium by *Shigella*, does not take place at the normal temperature but is induced at elevated temperatures (Hale 1991). However, some genes responsible for cellular processes under normal conditions may, in addition, take part in bacterial survival during stressful conditions. For example, diadenosine tetraphosphate, encoded by the *apaH* gene, controls the timing of cell division in *E. coli* (Nishimura et al. 1997) and also signals the heat and oxidative stress responses of *E. coli* (Lee et al. 1983).

**Virulence characteristics**

Virulence characteristics, also called virulence factors, are bacterial products that contribute to virulence. When the virulence factors are deleted, the pathogenic potential of the microbe is impaired. In addition to virulence factors, pathogens possess several virulence mechanisms--strategies that promote pathogenicity and the maintenance of their niche.
Bacterial adherence to host cells is of special importance for the persistence of bacteria in the oral cavity, small intestine, and urinary tract, surfaces that are cleansed by body fluids. Adherence is mediated by fimbriae, hair-like protein structures that extend from the bacterial cell surface and bind to host cell molecules, or bacterial surface proteins that mediate tight binding between bacteria and host cells (Soto & Hultgren 1999). The formation of a biofilm, a tight coaggregation of bacteria adhering to each other and to the host surface, helps the bacteria to colonize, to resist the host defense and antimicrobial agents, and also provides good sites for receiving nutrients (Beveridge et al. 1997).

Bacteria have several virulence strategies to evade of the host response. Many mucosal pathogens, such as oral streptococci, produce immunoglobulin A (IgA) proteases to cleave secretory immunoglobulin A (S1gA), which is produced by host cells to inhibit bacterial adherence (Marcotte & Lavoie 1998). Capsule formation may protect bacteria from the host’s inflammatory response and from phagocytosis. Encapsulated *Haemophilus influenzae* strains, especially those with a type b capsule, cause life-threatening and sometimes fatal infections, whereas nonencapsulated (nontypeable) *H. influenzae* strains rarely do so (Gildsdorf 1998). Bacteria can avoid the host response by antigenic variation: altering the surface proteins which serve as targets for host’s antibodies (Brunham et al. 1993).

Virulence factors that damage the host tissues are exotoxins, endotoxins, and hydrolytic enzymes. Exotoxins may have general cytotoxic effects, or they may be targeted at a certain host cell type as are neurotoxins, leukotoxins, or hepatotoxins (Salyers & Whitt 1994). Virulence due to exotoxin release is typical for *Corynebacterium diphtheriae*, *Vibrio cholerae*, and *Clostridium tetani* (Salyers & Whitt 1994, Casadevall & Pirofski 1999). Endotoxin refers to lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria (Proctor et al. 1995). Instead of being directly toxic to host cells, LPS provokes the host response by stimulating the host cells to produce endogenous substances, such as cytokines (Proctor et al. 1995, Helander et al. 1996).

The host response may be activated, in addition to toxins, also by autoimmune modulators of bacteria. This response is observed in rheumatic fever when streptococcal M proteins of *Streptococcus pyogenes* cross-react with heart tissue cells (Salyers & Whitt 1994). Heat shock protein (HSP) production is a defense mechanism against various environmental stresses for both eukaryotic and prokaryotic cells (Ellis 1996), but it may also play an important role in the virulence of microbial pathogens (Lathigra et al. 1991). Bacterial HSPs are proteins conserved during evolution and they show a high homology between different
bacterial species and also with human HSP (Ando et al. 1995, Multhoff et al. 1998, Zügel & Kaufmann 1999). Therefore, the bacterial HSPs may cross-react with the host and evoke an autoimmune response, such as suggested in rheumatoid arthritis or insulin-dependent diabetes mellitus (Kaufmann 1990, Zügel & Kaufmann 1999). Recently, \textit{Chlamydia pneumoniae} and human HSP60s have been found to activate human vascular cells by mechanisms that contribute to atherogenesis (Kol et al. 1999).

**Characteristics associated with virulence of \textit{A. actinomyctetemcomitans}**

The wide range of virulence characteristics of \textit{A. actinomyctemcomitans} ensures its survival in the oral cavity and enhances its capacity to cause periodontal destruction (for reviews see Wilson & Henderson 1995, Fives-Taylor et al. 1999). Table 1 summarizes the various virulence characteristics of \textit{A. actinomyctemcomitans} and gives a general overview of their biological activities. In addition to their virulence characteristics, bacteria possess several mechanisms to promote pathogenicity or interfere with host tissue repair and healing after infection. What follows concentrates on those \textit{A. actinomyctemcomitans} virulence-associated characteristics and mechanisms that have been objects of the present studies.

**Intraspecies diversity**

Differences have been observed in the detection frequency and pathogenic potential of certain \textit{A. actinomyctemcomitans} strains in various periodontal conditions. \textit{A. actinomyctemcomitans} serotype b strains are associated with both LJP and adult periodontitis (Zambon et al. 1983, Asikainen et al. 1991). Elevated pathogenic potential has been suggested for some oral \textit{A. actinomyctemcomitans} clones by their association with conversion from a healthy state to periodontal destruction (DiRienzo et al. 1994, Bueno et al. 1998) or association with advanced periodontitis (Asikainen et al. 1995, He et al. 1998, Macheleidt et al. 1999). On the contrary, certain \textit{A. actinomyctemcomitans} serotype c strains or AP-PCR genotypes, since they are frequently isolated from periodontally healthy subjects, may possess decreased virulence (Asikainen et al. 1991, 1995, Macheleidt et al. 1999). Additionally, adherence to epithelial cells (Meyer & Fives-Taylor 1994) and the capacity to invade human
cells (Meyer et al. 1991, Lepine et al. 1998) are observed to vary between A. actinomycetemcomitans strains.

**Serotypes and nonserotypeability**

The serotypes of A. actinomycetemcomitans are defined by the manner in which the carbohydrate cell-surface antigens react with antisera against A. actinomycetemcomitans (Slots et al. 1982, Zambon et al. 1984). Serotype-specific antigens of A. actinomycetemcomitans comprise more than 90% of neutral sugars and a small amount of fatty acids (Amano et al. 1989, Shibuya et al. 1991) and are suggested to reside in the O antigen of LPS. Table 2 presents studies on serotype-specific antigens of A. actinomycetemcomitans.

According to current knowledge, A. actinomycetemcomitans strains that react with none of the antisera raised against the five serotypes (a through e) (Saarela et al. 1992, Gmüer et al. 1993) are defined as nonserotypeable. Nonserotypeable strains have been detected by an immunodiffusion method with serotype-specific polyclonal rabbit antisera against the five serotypes (Saarela et al. 1992), by indirect immunofluorescence with serotype-specific monoclonal antibodies (Gmüer et al. 1993), and by immunostaining with human sera containing high antibody titers to A. actinomycetemcomitans (Gmüer et al. 1993). No observations have been made on the serotype switching of A. actinomycetemcomitans during the course of a periodontal infection (Saarela et al. 1992, 1999). In some other bacterial species, however, antigenic variation is common; *Borrelia hermsii* switches the serotype during relapsing fever (Barbour et al. 1991) and *H. influenzae* alters the immunodominant epitopes during colonization (Foxwell et al. 1998). It remains unknown whether the nonserotypeable A. actinomycetemcomitans strains are variants of the serotypeable strains or represent a new serotype. Additionally, the bacterial mechanisms involved in allowing an A. actinomycetemcomitans strain to remain nonserotypeable and unrecognized by the host, are not yet clear.
Table 1. Virulence-associated characteristics of *A. actinomycetemcomitans*.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Biological activity or effects on the host</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristics promoting colonization and growth</strong></td>
<td>Mediation of bacterial cell adherence and attachment to epithelial cells, to other bacteria, and to extracellular matrix proteins</td>
<td>Meyer &amp; Fives-Taylor 1994</td>
</tr>
<tr>
<td>Adhesins (fimbriae, extracellular membranous vesicles, extracellular amorphous material)</td>
<td>Inhibition of the growth or killing of other bacterial species, e.g., <em>Streptococcus sanguis</em> and <em>Actinomyces viscosus</em>, and other <em>A. actinomycetemcomitans</em> strains</td>
<td>Hammond et al. 1987</td>
</tr>
<tr>
<td>Bacteriocin (actinobacillicin)</td>
<td>Aid in bacteria penetrating eukaryotic cells</td>
<td>Sreenivasan et al. 1993</td>
</tr>
<tr>
<td>Invasins</td>
<td>Enhancement of iron utilization from the environment or the host</td>
<td>Winston et al. 1993, Willemsen et al. 1997</td>
</tr>
<tr>
<td>Iron-repressible protein, iron-regulated protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immunoinhibitory characteristics</strong></td>
<td>Disruption and inhibition of PMN chemotaxis</td>
<td>Van Dyke et al. 1982</td>
</tr>
<tr>
<td>Chemotaxis inhibitor</td>
<td>Inhibition of complement activation, downregulation of B-cell response</td>
<td>Tolo &amp; Helgeland 1991, Mintz &amp; Fives-Taylor 1994</td>
</tr>
<tr>
<td>Production of Fc-binding proteins</td>
<td>Inhibition of PNMs’ ability to produce antibacterial compounds, or intrinsic resistance to antibacterial compounds</td>
<td>Ashkenazi et al. 1992</td>
</tr>
<tr>
<td>Resistance to killing and phagocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production of Ig proteases</td>
<td>Degradation of IgG, serum IgA, and IgM</td>
<td>Gregory et al. 1992</td>
</tr>
<tr>
<td>Capsular polysaccharide</td>
<td>Resistance to phagocytosis by PMNs, reduction in complement-dependent response by PMNs, increase in bone resorption</td>
<td>Yamaguchi et al. 1995, 1998, Wilson et al. 1985</td>
</tr>
<tr>
<td>A variety of surface antigens</td>
<td>Aid in avoiding host antibodies and antibody production</td>
<td>Ebersole et al. 1996,</td>
</tr>
<tr>
<td>Immunosuppressive factors</td>
<td>Inhibition of DNA, RNA, and protein synthesis in T-lymphocytes and production of IgG and IgM in B-lymphocytes, downregulation of cytokine production</td>
<td>Nakashima et al. 1997</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Leukotoxin</td>
<td>Killing of PMNs, monocytes, and T-lymphocytes</td>
<td>Sugai et al. 1998, Shenko et al. 1999, Mayer et al. 1999</td>
</tr>
<tr>
<td>Toxin from culture supernatant</td>
<td>Fibroblast growth inhibition, apoptosis in B-lymphocytes</td>
<td>Baehni et al. 1979, Tsai et al. 1979, Mangan et al. 1991</td>
</tr>
<tr>
<td>Epitheliotoxin</td>
<td>Aid in penetrating the sulcular epithelium</td>
<td>Helgeland &amp; Nordby 1993, Ohguchi et al. 1998</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Bone resorption, local Schwartzman reaction, and tissue inflammation by inducing inflammatory mediator production from host cells</td>
<td>Birkedal-Hansen et al. 1982</td>
</tr>
<tr>
<td>GroEL-like heat shock protein</td>
<td>Bone resorption, epithelial-cell cytotoxicity and proliferation</td>
<td>Kiley &amp; Holt 1980, Saglie et al. 1990</td>
</tr>
<tr>
<td>Surface-associated material</td>
<td>PMN cytotoxicity, fibroblast anti-proliferativity, bone resorption</td>
<td>Kirby et al. 1995, Goulhen et al. 1998</td>
</tr>
<tr>
<td>Hemolysin</td>
<td>Lysing of human erythrocytes</td>
<td>Meghji et al. 1992, White et al. 1995</td>
</tr>
<tr>
<td>Proteinase production</td>
<td>Degradation of host proteins</td>
<td>Kimizuka et al. 1996</td>
</tr>
<tr>
<td>Extracellular membranous vesicles</td>
<td>Mediation of endotoxic, leukotoxic, and bone resorption activities</td>
<td>Mayrand et al. 1996</td>
</tr>
</tbody>
</table>

**Table 2.** Serotype-specific antigens in *A. actinomycetemcomitans.*

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>Antigen extraction methods</th>
<th>Antigen characterization and comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b, c</td>
<td>Sonication and ethanol precipitation of cell-free culture supernatant</td>
<td>Mannose-containing carbohydrate, polysaccharides on the cell surface.</td>
<td>Slots et al. 1982, Zambon et al. 1984, 1988</td>
</tr>
<tr>
<td>a, b, c</td>
<td>Sonicated whole cells</td>
<td>Carbohydrate, possibly capsular polysaccharide. Serotype-specific antigens are the immunodominant antigens in periodontitis patients with high antibody levels against <em>A. actinomycetemcomitans</em> of serotypes b and c, but not of serotype a.</td>
<td>Califano et al. 1989, 1991, 1992</td>
</tr>
<tr>
<td>a, b, c</td>
<td>Supernatant of autoclaved whole cells</td>
<td>Polymer consisting of a disaccharide repeating unit (serotype b) and closely related repeating units (serotypes a, and c). Serotype-specific antigens differ from O polysaccharides of LPS.</td>
<td>Amano et al. 1989, Shibuya et al. 1991</td>
</tr>
<tr>
<td>b</td>
<td>Phenol-water extraction of LPS from whole cells</td>
<td>Serotype-specific antigen is in the polysaccharide of LPS.</td>
<td>Wilson &amp; Schifferle 1991</td>
</tr>
<tr>
<td>b</td>
<td>Boiled and sonicated whole cells</td>
<td>Serotype-specific antigen, the immunodominant antigen in juvenile periodontitis patients, resides in the high-molecular-mass carbohydrate rich LPS fraction.</td>
<td>Sims et al. 1991, Page et al. 1991</td>
</tr>
<tr>
<td>b</td>
<td>Sonicated whole cells</td>
<td>Serotype-specific antigen is a high-molecular-weight polysaccharide separable from LPS. Antigen of capsular material.</td>
<td>McArthur et al. 1996</td>
</tr>
<tr>
<td>a, b, c, d, e</td>
<td>Phenol extraction of LPS and purification of O polysaccharide</td>
<td>Polymer consisting of disaccharide (serotypes a, c, and e), trisaccharide (serotype b), and tetrasaccharide (serotype d) repeating units. Serotype-specific antigens are in the O polysaccharide of LPS.</td>
<td>Perry et al. 1996a, 1996b</td>
</tr>
</tbody>
</table>
Lipopolysaccharide

LPS consists of a lipid A portion, a core, and a polysaccharide (O antigen) (Hitchcock et al. 1986). The highly variable carbohydrate O antigen is the immunodominant antigen in several bacterial species and presents to the host various antigenic structures (Proctor et al. 1995, Helander et al. 1996). The lipid A portion of LPS is highly conserved, exhibiting the smallest structural variation in LPS, with different LPS core structures existing mainly between different genera (Helander et al. 1996).

The serotype b-specific antibody binding to A. actinomycetemcomitans is mediated through the LPS O antigen (Page et al. 1991, Wilson & Schifferle 1991). LPS O polysaccharide is of a unique chemical composition in each of the reference strains representing all five A. actinomycetemcomitans serotypes (Perry et al. 1996a, 1996b), supporting the view that in all A. actinomycetemcomitans serotypes the serotype-specific antigens are localized in the O-antigenic polysaccharide of LPS. However, some controversy exists, with studies suggesting that serotype determinants in A. actinomycetemcomitans are polysaccharides structurally differing from the O antigen, or that A. actinomycetemcomitans may produce multiple serotype-specific polysaccharides (Amano et al. 1989, Shibuya et al. 1991, McArthur et al. 1996). Among Salmonella species, the presence or length of the O antigen defines the virulence of the bacteria (Valtonen 1970, Helander et al. 1996). No differences, however, have been seen in the periodontal disease state of subjects with nonserotypeable A. actinomycetemcomitans strains compared to those with serotypeable strains (Asikainen et al. 1991, 1995, Saarela et al. 1999).

Besides acting as an antigenic structure to the host, A. actinomycetemcomitans LPS stimulates cytokine production from PMNs, monocytes, and macrophages (Saglie et al. 1990, Agarwal et al. 1995, Yoshimura et al. 1997). Additionally, LPS alterations have been reported to be responsible for antimicrobial resistance in Pseudomonas aeruginosa and E. coli (Leying et al. 1992, Tarr et al. 1998). No data are available on the effects of LPS alterations in the antimicrobial susceptibility of A. actinomycetemcomitans strains.

Outer membrane proteins

The major outer membrane proteins (OMPs) of A. actinomycetemcomitans are largely similar in A. actinomycetemcomitans serotypes a, b, and c (DiRienzo & Spieler 1983, Bolstad et al. 1990, Muller et al. 1990, van Steenbergen et al. 1994). One nonserotypeable A. actinomycetemcomitans strain has exhibited OMP patterns similar to those of serotypeable
strains (van Steenbergen et al. 1994). However, some differences in OMP patterns have recently been found between four A. actinomycetemcomitans strains with rough and smooth colony morphologies (Haase et al. 1999). Growth of A. actinomycetemcomitans in 5% CO2 in air or in anaerobiosis may affect its cell-surface ultrastructure and surface protein expression (Scannapieco et al. 1987). Its OMPs have been identified as targets for immunoglobulin G (IgG) antibodies in sera from LJP patients (Bolstad et al. 1990, Wilson & Hamilton 1995), and as proteins binding to the Fc portion of immunoglobulins (Mintz & Fives-Taylor 1994).

OMP profiles show antigenic heterogeneity among subtypes of nontypeable H. influenzae and can be used for serotyping the isolates (Murphy & Apicella 1985). The typeability of H. influenzae, as seen by antisera reacting with the bacterial capsule (Gilsdorf 1998), however, is different from the serotypeability of A. actinomycetemcomitans, which is seen when antisera react with carbohydrate cell-surface antigens (Slots et al. 1982, Zambon et al. 1984). Therefore, the A. actinomycetemcomitans OMPs may not be likely candidates for the differentiation between serotypeable and nonserotypeable strains. However, little information is available on the OMPs of nonserotypeable A. actinomycetemcomitans strains.

**Polymorphism of genes**

In A. actinomycetemcomitans, genes responsible for leukotoxic (Lally et al. 1989) and cytotoxic (Mayer et al. 1999) activities have been characterized. Genes of A. actinomycetemcomitans encoding for proteins essential for its survival in environmental changes, such as reduced iron availability (Winston et al. 1993) or elevated temperature (Winston et al. 1996, Minami et al. 1998), have been cloned and sequenced. No information is available on other stress-associated gene expression such as apaH (Farr et al. 1989) in A. actinomycetemcomitans.

**Leukotoxin**

Leukotoxin is among the most studied virulence factors of A. actinomycetemcomitans. Its leukotoxin is a member of the repeats-in-toxin (RTX) exoprotein family of pore-forming hemolysins/leukotoxins (Welch 1995) and is specifically cytotoxic to human polymorphonuclear leukocytes (PMNs), monocytes, and T-lymphocytes (Baehni et al. 1979, Tsai et al. 1979, Mangan et al. 1991). It has recently been shown to induce apoptosis in promyelocytic cell line HL-60 (Korostoff et al. 1998). A high production of leukotoxin is associated with A. actinomycetemcomitans strains isolated from patients with LJP (Zambon et
al. 1996, Bueno et al. 1998). All A. actinomycetemcomitans strains seem to have genes that code for leukotoxin (Poulsen et al. 1994); a deletion in the leukotoxin gene promoter region leads to expression of increased leukotoxic activity (Brogan et al. 1994). This deletion of the leukotoxin gene promoter structure of oral A. actinomycetemcomitans is rare (Brogan et al. 1994) and has not been detected in strains from northern Europe (Haubek et al. 1995, Asikainen et al. 1997). Little information exists as to the leukotoxin gene promoter structure of A. actinomycetemcomitans strains from nonoral infections.

**Heat shock proteins**

HSPs are produced as a protection against stress (Ellis 1996), but they also play a role under normal conditions during the cell cycle, development, and differentiation (Bukau & Horwich 1998). HSPs may additionally function as molecular chaperones ensuring that protein assembly into higher order structures occurs correctly (Ellis 1996).

Several authors have reported in A. actinomycetemcomitans the presence of HSPs, including GroEL-like (HSP60) and DnaK-like (HSP70) proteins (Koga et al. 1993, Løkensgaard et al. 1994, Nakano et al. 1995, Hinode et al. 1998, Goulhen et al. 1998). Protein homologous to GroEL-like HSP found in the surface-associated material of A. actinomycetemcomitans has osteolytic activity by murine bone resorption assay (Kirby et al. 1995). Purified native GroEL-like HSP from A. actinomycetemcomitans promotes epithelial-cell proliferation at lower HSP concentrations, but has a toxic effect on epithelial cells at higher HSP concentrations (Goulhen et al. 1998). There are to the best of our knowledge, no previous studies comparing HSP expression between several clinical strains of any bacterial species, and, thus no information is available on the relation of HSP expression to the course of an infection.

As mentioned above, bacterial and human HSPs share high sequence homology. Antibodies raised against GroEL-like proteins from A. actinomycetemcomitans, P. gingivalis, and B. forsythus show a high level of cross-reactivity to each other (Hinode et al. 1998), and may also react with HSP65 from H. influenzae and E. coli (Nakano et al. 1995). Autoimmune mechanisms may play a role in gingivitis and periodontitis. Salivary IgA antibodies against mycobacterial HSP65 are significantly increased in patients with gingivitis (Schett et al. 1997), and the proliferative response of peripheral blood mononuclear cells from periodontitis patients to HSPs is lowered (Petit et al. 1999).
Antimicrobial resistance

Antimicrobial resistance of a pathogen may be associated with its virulence. Antimicrobial resistance may promote the persistence of a pathogen in the infected host during antimicrobial treatment and lead to prolonged or life-threatening infections. Resistance may also contribute to the quick and unlimited spread of a pathogen in a population. Approximately 30% of oral A. actinomycetemcomitans are resistant to benzylpenicillin (Slots et al. 1980b, Walker et al. 1985), the most commonly used antimicrobial agent in treatment of oral infections. In severe nonoral A. actinomycetemcomitans infections the antimicrobial susceptibility of the pathogen plays a crucial role in the recovery of the patient. The data on antimicrobial susceptibility of nonoral A. actinomycetemcomitans strains are restricted to separate case reports from laboratories around the world.

Production of betalactamase enzyme is the major penicillin resistance mechanism, especially among gram-negative bacteria (Hedberg & Nord 1996), but it has not been observed in A. actinomycetemcomitans strains (Slots et al. 1980b). New or altered penicillin-binding proteins on the bacterial cell-surface may account for the non-enzymatic penicillin resistance of A. actinomycetemcomitans, as has been observed among strains of H. influenzae (Clairoux et al. 1992). Poor permeability of the outer membrane may be responsible for the antimicrobial resistance in gram-negative organisms. Additionally, an active efflux mechanism may transport the drug from the cytoplasm out of the bacterial cell, as observed in E. coli and P. aeruginosa (Nikaido 1998).

Stimulation of the epithelial response

In periodontal destruction, bacterial substances mainly interact with mononuclear phagocytic cells and fibroblasts (Page & Schroeder 1977, World Workshop in Periodontics 1996). However, cells of junctional epithelium and periodontal pocket epithelium are the first host cells to face the bacteria during the microbial challenge in periodontitis (Schroeder 1996). Bacteria and bacterial products in the periodontal pocket evoke the host defense and stimulate epithelial cells to produce enzymes such as matrix metalloproteinases (Birkedal-Hansen et al. 1993, Uitto et al. 1998), cytokines such as interleukins and tumor necrosis factor-α (Kornman et al. 1997, Okada & Murakami 1998), or antimicrobial peptides such as β-defensins (Weinberg et al. 1998). The epithelial-cell response to A. actinomycetemcomitans includes stimulation of chymotrypsin-like enzyme secretion (Firth et al. 1996), increased secretion of
interleukin-8 and intercellular adhesion molecule-1 (Huang et al. 1998), and activity promoting epithelial-cell proliferation or death (Meghji et al. 1992, Goulhen et al. 1998).

Cell proliferation, when occurring in the apical direction in the junctional epithelium, is a key mechanism in the formation and deepening of the periodontal pocket. The development of a model for junctional epithelium (Pan et al. 1995) by culturing periodontal ligament epithelial cells, isolated from epithelial cell rests of Malassez (Brunette et al. 1976), has made possible in vitro studies on junctional epithelium. A. actinomycetemcomitans HSP60 increases epithelial-cell proliferation and therefore may promote the progression of periodontal disease (Goulhen et al. 1998). On the other hand, inhibition of this epithelial-cell proliferation by A. actinomycetemcomitans (Meghji et al. 1992, Goulhen et al. 1998) may delay healing and repair of host tissues after periodontal infection. Information on the proliferative effects of clinical A. actinomycetemcomitans strains on epithelial cells is limited, and it is not known whether heat-stressed clinical strains provoke responses similar to those of purified HSP.
AIMS OF THE STUDY

The hypothesis of the studies included in this thesis was that differences exist in the virulence characteristics among *A. actinomycetemcomitans* strains. Nonserotypeable *A. actinomycetemcomitans* strains and *A. actinomycetemcomitans* strains from nonoral infections were assumed to demonstrate higher virulence than that of serotypeable *A. actinomycetemcomitans* strains or strains originating in the periodontally healthy oral cavity.

The general aim of the present study was to find *A. actinomycetemcomitans* strains with elevated virulence properties by determining why some *A. actinomycetemcomitans* strains remain nonserotypeable, by characterizing *A. actinomycetemcomitans* strains from nonoral infections, and by studying the heat-induced stress response of *A. actinomycetemcomitans* strains in relation to the *in vitro* effects on epithelial-cell viability.

The specific questions were:
1. Do nonserotypeable and serotypeable *A. actinomycetemcomitans* strains have a common origin? (I)
2. In comparison with serotypeable strains, do nonserotypeable *A. actinomycetemcomitans* strains have cell-surface alterations that contribute to their antigenicity? (II)
3. Do certain characteristics of *A. actinomycetemcomitans* strains from nonoral infections differ from those of strains from the oral cavity? (III)
4. Does heat shock protein expression contribute to the variable ability of clinical *A. actinomycetemcomitans* strains to induce epithelial-cell proliferation or death? (IV)
MATERIAL AND METHODS

Subjects and bacterial strains

A total of 170 *A. actinomycetemcomitans* strains and 6 reference strains of other bacterial species were used in the present studies (Table 3).

Table 3. Number of bacterial strains used in Studies I, II, III, and IV.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinobacillus actinomycetemcomitans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral, serotypeable</td>
<td>18*</td>
<td>12</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Oral, nonserotypeable</td>
<td>75</td>
<td>12</td>
<td>-</td>
<td>1</td>
</tr>
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<td>Nonoral, serotypeable</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>Nonoral, nonserotypeable</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><strong>Control strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral, serotypeable</td>
<td>-</td>
<td>3</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Oral, nonserotypeable</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td><strong>Reference strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>H. aphrophilus</em></td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>96</td>
<td>36</td>
<td>80</td>
<td>12</td>
</tr>
</tbody>
</table>

1 Control strains are human isolates from the collection of the Institute of Dentistry, University of Helsinki.

2 Reference strains are from international (ATCC, NCTC) strain collections in addition to acknowledged *A. actinomycetemcomitans* reference strains from the Institute of Dentistry, University of Helsinki (IDH) and a kind gift from Professor J. Slots (JP2).

Studies on the nonserotypeability of *A. actinomycetemcomitans* (I, II)

The study material comprised 75 nonserotypeable oral *A. actinomycetemcomitans* isolates from 34 unrelated subjects (age-range 14-68 years). These isolates came from our strain collection of more than 1,300 *A. actinomycetemcomitans* strains at the Institute of Dentistry, University of Helsinki, and comprised all nonserotypeable *A.
actinomycetemcomitans strains available. Each of the 34 subjects contributed 1 to 12 (mean 2.7) A. actinomycetemcomitans isolates, of which 1 to 7 (mean 2.2) were serologically nontypeable. Of the 34 subjects, 6 (age-range 31-57 years) harbored both serotypeable \((n = 18)\) and nonserotypeable \((n = 12)\) isolates--each contributed 2 to 6 isolates--and these isolates were used to compare the serotypeable and nonserotypeable isolates from the same subjects. All of the isolates originated in samples of subgingival plaque and saliva, and samples from the tongue surface or oral mucosa. Three A. actinomycetemcomitans ATCC strains (ATCC 29523, ATCC 43718, and ATCC 33384) were included in the material as reference strains, and three clinical strains from LJP or adult periodontitis were included as control strains. In addition, Haemophilus influenzae ATCC 49247 and ATCC 49766, H. aphrophilus ATCC 13252 and NCTC 5906, Staphylococcus aureus ATCC 25923, and E. coli ATCC 25922 were included as reference strains in the antimicrobial susceptibility testing.

Studies on nonoral A. actinomycetemcomitans (III)

The bacterial collection comprised 52 A. actinomycetemcomitans strains from 51 subjects (ages unknown) with various nonoral infections and 21 oral strains from 21 subjects (age-range 14-71 years) with periodontitis or with healthy periodontium. The A. actinomycetemcomitans strains from nonoral infections were obtained from geographically distant locations. One source was the Culture Collection at the University of Gothenburg in Sweden (Sweden, \(n = 24\); Austria, \(n = 1\); Germany, \(n = 2\); and the United States, \(n = 1\)), another source was international research groups with published or unpublished data on nonoral A. actinomycetemcomitans infections (Iceland, \(n = 4\); Belgium, \(n = 2\); New Zealand, \(n = 6\); and Taiwan, \(n = 4\)), and a third source was patients with unpublished A. actinomycetemcomitans infections from hospitals in Finland \((n = 8)\).

The 21 oral A. actinomycetemcomitans strains were selected from our culture collection to match the serotype and genotype distributions of those nonoral strains, and they originated in subjects with periodontitis, gingivitis, or healthy periodontium. A. actinomycetemcomitans strain JP2 was used as a positive control strain in the analysis of leukotoxin gene promoter structure. H. influenzae ATCC 49247 and ATCC 49766, H. aphrophilus ATCC 13252 and NCTC 5906, S. aureus ATCC 25923, and E. coli ATCC 25922 were included as reference strains in the antimicrobial susceptibility testing.
Studies on the heat shock response of A. actinomycetemcomitans and its effects on epithelial-cell proliferation and cytotoxicity (IV)

The study bacteria comprised 12 A. actinomycetemcomitans strains from 11 subjects. The clinical strains were selected from the previous studies (I, II, III) to represent various oral and nonoral infections, and the healthy oral cavity. Reference strains (ATCC 29523, ATCC 43718, ATCC 33384, IDH 781, IDH 1705, and JP2), representing all five A. actinomycetemcomitans serotypes, were also included.

Methods

Culture and identification (I, II, III, IV)

A. actinomycetemcomitans strains were grown on Trypticase soy-serum-bacitracin-vancomycin agar plates (Slots 1982b) and incubated in 5% CO₂ in air at 37°C for 2 to 3 days. Isolates were identified as A. actinomycetemcomitans if they presented a typical colony morphology, a positive catalase reaction, and a negative result for lactose fermentation (Asikainen et al. 1986, Saarela et al. 1992). Subcultures started from a single colony were preserved in 20% skim milk at -70°C until used.

Serotyping (I, III)

Antigens for serotyping were prepared from A. actinomycetemcomitans cells grown in 5 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, MI) in 5% CO₂ in air at 37°C for 2 to 3 days. Bacterial cells were collected by centrifugation, washed once with distilled water, suspended in 150 µl of 0.9% NaCl, and autoclaved at 120°C for 15 min. Undiluted serotype-specific rabbit antisera made against whole cells of A. actinomycetemcomitans serotypes a to e (Saarela et al. 1992) were used in an immunodiffusion assay carried out in 1.2% Noble agar (Difco) (Saarela et al. 1992). A. actinomycetemcomitans strains representing the five serotypes were ATCC 29523 (serotype a), ATCC 43718 (serotype b), ATCC 33384 (serotype c), IDH 781 (serotype d), and IDH 1705 (serotype e).
Polymerase chain reaction (PCR) assays (I, III)

**DNA extraction for PCR amplifications.** Chromosomal DNA used as a template in PCR amplifications was extracted from *A. actinomycetemcomitans* strains by a modification of the method of Moncla and coworkers (1990). Briefly, after lysozyme incubation and 10% SDS and proteinase K digestions, proteins were removed by extractions with phenol:chloroform and chloroform. DNA was precipitated by using sodium acetate, dried under vacuum, and dissolved in Tris-EDTA buffer (Saarela et al. 1995).

**Arbitrarily primed PCR (AP-PCR) genotyping.** The random sequence oligonucleotide OPA-13 (5'-CAGCACCCAC-3') (Operon Technologies, Inc., Alameda, CA) was used as a primer for all *A. actinomycetemcomitans* isolates (Slots et al. 1993, Asikainen et al. 1995). Two additional primers, OPA-03 (5'-AGTCAGCCAC-3') and OPA-07 (5'-GAAACGGGTG-3'), were used for selected isolates indistinguishable by OPA-13 primer (Slots et al. 1993). AP-PCR was performed as previously described (Asikainen et al. 1995). Amplification products were analyzed electrophoretically in 1% (wt/vol) agarose gel containing ethidium bromide (0.5 mg/ml) and visualized under ultraviolet light.

**apaH PCR amplification and restriction analysis.** Primers used in the PCR amplification of the *apaH* gene were 5’-ATTTAATCGGCGACCTGCAC-3’ and 5’-TGTCTTTCCAACGTAGCATG-3’ (Saarela et al. 1998). PCR amplification was performed as previously described (Saarela et al. 1998). Amplification products were characterized by restriction analysis by use of restriction endonucleases *SphI* and *NheI* as previously described (Saarela et al. 1998).

**Analysis of leukotoxin promoter structure by PCR.** The primer pair 5’-ATA TTA AAT CTC CTT GT-3’ and 5’-ACC TGA TAA CAG TAT T-3’ (Brogan et al. 1994) was used to amplify a DNA fragment from the leukotoxin promoter region of *A. actinomycetemcomitans* strains. PCR amplification was performed as described earlier (Asikainen et al. 1997). A positive control, DNA of highly leukotoxic *A. actinomycetemcomitans* strain JP2 (an amplicon of 470-bp) (Brogan et al. 1994) and a negative control (reaction mixture without template) were included in each PCR amplification. Amplification products were analyzed electrophoretically in 1% (wt/vol) agarose gel containing ethidium bromide (0.5 mg/ml) and visualized under ultraviolet light.
Antimicrobial susceptibility testing (II, III)

The minimum inhibitory concentrations (MICs) of six antimicrobial agents for *A. actinomycetemcomitans* strains were determined by the agar dilution method with *Haemophilus* test medium according to established methods and to the instructions of the National Committee for Clinical Laboratory Standards (NCCLS) (1998a, 1998b). The six antimicrobial agents included benzylpenicillin, amoxicillin, tetracycline, metronidazole, azithromycin, and trovafloxacin.

Preparation of *A. actinomycetemcomitans* cell-surface structures (II)

**Isolation of *A. actinomycetemcomitans* outer membrane proteins (OMPs).** Outer membrane proteins of the bacterial strains were prepared by the modified methods of Filip et al. (1973) and of Chen and Wilson (1990). *A. actinomycetemcomitans* strains were grown on Trypticase soy agar (Difco) plates in 5% CO₂ in air at 37°C for 2 to 3 days. Bacterial colonies were suspended in phosphate-buffered saline (PBS, pH 7.4) and disrupted with a sonicator (Sonifier Branson 250) using a 50% duty cycle at output 6 for 4.5 min. Intact cells and insoluble debris were removed by centrifugation at 3,000 × g for 10 min. The total membrane fraction in the supernatant was recovered by centrifugation at 100,000 × g for 1 h, and treated with 1% Sarkosyl (sodium-lauroyl sarcosinate, Sigma Chemical Co., St. Louis, MO) for 1 to 4 h at room temperature. The Sarkosyl solution was centrifuged at 100,000 × g for 1 h to collect the sarkosyl-insoluble outer membrane fraction, and the pellet comprising the outer membrane complex (OMC) was suspended in distilled water.

**Isolation of *A. actinomycetemcomitans* lipopolysaccharide (LPS).** The LPS fraction was prepared from OMC (Chen & Wilson 1990): part of the OMC fraction was dispersed in lysing buffer (5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.125 M Tris-Cl, pH 6.8) and solubilized by incubation at 100°C for 10 min. The material was cooled to room temperature, 10 µl of proteinase K (10 mg/ml) was added, and the solution was incubated at 60°C for 60 min.

Heat shock and fractionation of *A. actinomycetemcomitans* cells (IV)

The bacteria were first grown on Trypticase soy agar (Difco) and then in Todd-Hewitt broth (Difco) supplemented with 1% yeast extract at 35°C in anaerobic jars filled with mixed gas (85% N₂, 10% H₂, 5% CO₂). For each *A. actinomycetemcomitans* strain, two agar plates
for the recovery of whole cells and two broth cultures (100 ml) for the fractionation of bacterial cells were either grown at 35°C (control, non-shocked) or at 43°C (heat-shocked) for 2 h. Whole bacterial cells were then collected from the agar plates and placed in tubes containing 1 ml of PBS.

The fractionation of *A. actinomycetemcomitans* cells was performed according to a modification of the method of Goulhen and coworkers (1998). Briefly, bacterial cells were exposed to osmotic shock with ice-cold distilled water for 5 min, the suspension was centrifuged, and the supernatant containing the periplasmic material was lyophilized. The cells were disrupted by ultrasonic treatment (Sonifier Branson 250) by using a 20% duty cycle at output 3 for 5 min and centrifuged at 6,000 × g for 15 min. The supernatant was then centrifuged at 200,000 × g for 2 h. The resulting supernatant was dialyzed against distilled water and contained the cytoplasmic material. The pellet was resuspended in distilled water and contained the cell membrane fraction (cytoplasmic and outer membranes).

**Analysis of OMPs, LPS, and heat shock proteins of *A. actinomycetemcomitans* (II, IV)**

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE was performed according to the modified Laemmli gel system (Laemmli 1970) on a vertical gel apparatus Mini-Protean II (Bio-Rad Laboratories, Richmond, CA). OMP electrophoresis was carried out with 12.5% resolving gels run at 200 V, and protein bands were visualized by staining with Coomassie brilliant blue R-250 (CBB) (Serva, Heidelberg, Germany). The LPS electrophoretic patterns were assessed with 15% resolving gels run at 200 V and visualized by silver staining (Hitchcock & Brown 1983). LPS from *E. coli* 0111:B4 (Sigma), *Salmonella* serotype Typhimurium SH 2183 (smooth) (Valtonen 1970), *Salmonella* serotype Typhimurium SH 5014 (Rb2) (Nurminen et al. 1976), *A. actinomycetemcomitans* Y4 (serotype b), and *A. actinomycetemcomitans* IDH 781 (serotype d) were used as references.

The protein concentration in the HSP samples was determined by a dye-binding assay (Bio-Rad kit) with BSA as a standard. HSP electrophoresis was carried out with 10% resolving gels run at 200 V for 45 min and proteins were stained with CBB. In all SDS-PAGE assays 4% stacking gels were used.

**Western immunoblotting.** *A. actinomycetemcomitans* LPSs were transferred onto a nitrocellulose membrane by a NovaBlot Electrophoretic Transfer kit with Multiphor II (Pharmacia LKB Biotechnology, Uppsala, Sweden). Western immunoblotting was performed
according to a modification of the method of Towbin's group (1979). The primary antibody was either rabbit antiserum against the one serotype which was detected from the serotypeable/nonserotypeable pair of A. actinomycetemcomitans strains from each study subject, or alternatively, a pool of rabbit antisera against serotypes a, b, and c (1:5,000 dilution). Both were incubated for 1 h. Serotype-specific rabbit antisera had been made against whole cells of A. actinomycetemcomitans serotypes a to e (Saarela et al. 1992). The membranes were then incubated with the secondary antibody: goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000 dilution, Sigma) for 1 h. Peroxidase staining (Sigma) was carried out to visualize the results of the immunoblotting.

A. actinomycetemcomitans cell fractions for the HSP immunodetection were transferred onto a nitrocellulose membrane by Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Western immunoblotting was performed by incubating the membrane with the primary antibodies, rabbit antiserum against native GroEL-like protein (1:1,000 dilution) from a heat-shocked A. actinomycetemcomitans strain ATCC 29522 (serotype b) (Hinode et al. 1996) and a commercial antibody against HSP70 protein (1:3,000 dilution, Dako Corporation, Carpinteria, CA) for 2 h as described previously (Hinode et al. 1996). The membrane was further incubated with the secondary antibody goat anti-rabbit IgG conjugated to alkaline phosphatase (1:3,000 dilution, Bio-Rad) for 1 h. The reactions were visualized by alkaline phosphatase staining. The strength of HSP expression in the immunoblots was measured by scanning and computer analysis (NIH Image). The computerized analysis of the GroEL expression in the membrane fraction ranged from 0 to 3,000 relative units for weak, from 3,000 to 5,000 units for moderate, and 5,000 units or more for strong expression.

Epithelial-cell growth and viability assays (IV)

Porcine periodontal ligament epithelial cells (PLE cells), which resemble the junctional epithelial cells in humans (Pan et al. 1995), were cultured in minimal Eagle medium containing 0.01% penicillin G (w/v), 0.1% gentamycin sulphate (v/v), 1.2% fungizone (w/v) (Gibco BRL; 30 µg amphotericin B and 24.6 µg sodium deoxycholate per ml final concentration), and 10% fetal bovine serum. Cells were cultured in 5% CO2 at 37°C for 2 to 4 days depending on the assay; to 50% confluency for proliferation assays and to 100% confluency for cytotoxicity assays. PLE cells were cultured for a further 24 to 48 h in the presence of A. actinomycetemcomitans whole-cell or membrane preparations (heat-shocked and controls). Proliferation assays were performed in the presence of serum to facilitate further epithelial-cell
growth, whereas cytotoxicity assays were performed in the absence of serum to prevent the epithelial cells from further growth. The number of viable PLE cells was studied by measurement of the conversion of tetrazolium salt into blue formazan with Cell Titer 96 kit (Promega, Madison, WI). The results were of optical density at 570 nm and reflected the amount of viable PLE cells. Cytotoxicity assays gave negative figures reflecting PLE cell loss, which were then converted to positive figures reflecting the number of dead cells.

**Statistical methods (III)**

The statistical significance for the differences between frequency distributions of nonoral *A. actinomycetemcomitans* serotypes, genotypes, and antimicrobial susceptibilities was determined by Chi-square statistics and Fisher’s exact test.
RESULTS AND DISCUSSION

Nonserotypeability of *A. actinomycetemcomitans* (I, II)

More than 90% of clinical *A. actinomycetemcomitans* strains are serotypeable. *A. actinomycetemcomitans* strains that remain nonserotypeable may exhibit elevated virulence by impeding detection by the host, since serotype-specific antigens are the immunodominant antigens in *A. actinomycetemcomitans* -positive periodontitis patients (Califano et al. 1989, 1991). During the course of an infection some pathogens alter their cell-surface structures to evade a host response (Barbour et al. 1991, Foxwell et al. 1998). Cell-surface alterations may contribute to the nonserotypeability of *A. actinomycetemcomitans*. If no cell-surface alterations are found, a new serotype may be present. Therefore, 75 nonserotypeable *A. actinomycetemcomitans* strains, all the nonserotypeable strains available, were characterized in Studies I and II.

Characterization of nonserotypeable *A. actinomycetemcomitans* strains

Nonserotypeable oral *A. actinomycetemcomitans* strains were characterized by use of AP-PCR genotyping and *apaH* restriction analysis. AP-PCR technique, a rapid method in the clonal analysis of *A. actinomycetemcomitans* (Slots et al. 1993), distinguished, among the 75 nonserotypeable strains originating in 34 subjects, eight AP-PCR genotypes (Fig. 1 from original publication I). Six of these eight AP-PCR genotypes were similar to those found in the previous studies (Asikainen et al. 1995, 1996, 1997) and thus corresponded to previously known serotypes or nonserotypeable isolates. Two AP-PCR genotypes within two subjects had not been detected previously.

The most common AP-PCR genotype among the nonserotypeable *A. actinomycetemcomitans* strains was genotype 3, as categorized by Asikainen et al. (1995), which corresponds to serotype c (Asikainen et al. 1995). The results obtained with the *apaH* restriction analysis showed the same trend as the previous findings (Saarela et al. 1998): strains with a serotype c-like restriction pattern predominate among nonserotypeable *A. actinomycetemcomitans* strains. When the nonserotypeable *A. actinomycetemcomitans* strains were grouped into AP-PCR genotypes, according to Asikainen et al. (1995, 1996), and
corresponding to different serotypes, the distribution was: "serotype a" in 6 subjects (18%), "serotype b" in 3 subjects (9%), "serotype c" in 21 subjects (62%) and "serotype d" in 1 subject (3%) (Table 1 from original publication I). Twelve A. actinomyces strains from three subjects remained "nonserotypeable" with AP-PCR. The present results obtained by AP-PCR genotyping of nonserotypeable A. actinomyces strains show a discrepancy with the previous reports on the serotype distribution of oral A. actinomyces. Among A. actinomyces strains isolated from 91 (Saarela et al. 1992) and 356 (Dogan et al. 1999a) Finnish subjects, serotypes a, b, and c are about equally represented (25%, 29%, and 23%-26%, respectively) and serotypes d and e are rare (3%-4% and 6%-10%, respectively). Taken together, the previous reports and present results show that the A. actinomyces AP-PCR genotype corresponding to serotype c is far more common in nonserotypeable strains than could be expected based on the general serotype distribution of oral A. actinomyces strains (62% versus 23%-26%). This result suggests that nonserotypeable A. actinomyces strains represent a population of strains that originally were serotypeable, and that serotype c strains may lose their ability to react with serotype-specific antiserum easier than do strains of the other serotypes. The results of Poulsen et al. (1994) also suggest that nonserotypeable strains originate from strains of known serotypes. Characterization of eight nonserotypeable A. actinomyces strains showed that these strains were distributed in different evolutionary lines of the A. actinomyces population, and they were genetically closely related to other strains of their respective clusters (Poulsen et al. 1994). However, the present results do not fully exclude the possibility that a new A. actinomyces serotype representing the serotype c-specific AP-PCR genotype will be found if a larger sample of nonserotypeable A. actinomyces strains is analyzed.

Two of the eight AP-PCR genotypes found in the present study among the nonserotypeable A. actinomyces strains were not known from the previous studies (Asikainen et al. 1995, 1996, 1997). One or both of these two genotypes, plus one previously found genotype among three nonserotypeable strains (Asikainen et al. 1995), may represent one or more novel A. actinomyces serotypes. The existence of new serotype(s) needs further study.

Multiple nonserotypeable A. actinomyces strains were isolated from 23/34 subjects participating in the study. These strains showed clonality per subject in 22/23, whereas one subject had two AP-PCR genotypes. Therefore, the present Finnish subjects harboring
nonserotypeable *A. actinomycetemcomitans* strains are most likely to have only one clone of the organism per subject, as previously observed among serotypeable *A. actinomycetemcomitans* strains from Finnish subjects (Asikainen et al. 1995, 1996) and recently also from Japanese subjects (He et al. 1998).

**Comparison of serotypeable and nonserotypeable *A. actinomycetemcomitans* strains from the same subjects**

Of the 34 subjects with nonserotypeable *A. actinomycetemcomitans* strains, six also harbored serotypeable strains. In each subject the serotypeable and nonserotypeable *A. actinomycetemcomitans* strains produced what seemed to be identical AP-PCR banding patterns. This unique material, 12 strains of serotypes a, b, and c and 12 nonserotypeable strains all simultaneously isolated from six subjects, inspired the testing of the material to see whether the cell-surface structures of nonserotypeable *A. actinomycetemcomitans* strains differed from those of serotypeable strains.

In Western immunoblotting with serotype-specific antisera, nonserotypeable *A. actinomycetemcomitans* strains of serotype b- and c-specific AP-PCR genotypes lacked the clear LPS profile that was present in the serotypeable strains from the same subjects (Fig. 3B and 3C from original publication II). This LPS profile was seen as a smear in the high-molecular-weight O-antigenic region of LPS in immunoblots of serotypeable strains only, although the SDS-PAGE/silver staining of the gels showed no apparent differences in the LPSs between serotypeable and nonserotypeable *A. actinomycetemcomitans* strains (Fig. 2 from original publication II). These results suggest that alterations in the LPS of the present nonserotypeable *A. actinomycetemcomitans* strains of serotype b- or c-specific AP-PCR genotypes has led to lack of reactivity with the serotype-specific antisera. The LPS alteration seems to be in the O antigen of LPS, since the serotypeable *A. actinomycetemcomitans* strains give typical O-antigenic profiles in the immunoblots. Our present results support the previous studies' findings that the serotype-specific structures of *A. actinomycetemcomitans* are in the long-chain high-molecular-weight O antigen of LPS (Page et al. 1991, Wilson & Shifferle 1991, Perry et al. 1996a, 1996b). In notable contrast to the previous and present results, some studies suggest that *A. actinomycetemcomitans* strains of serotypes a, b, and c produce multiple serotype-specific polysaccharides separable from the LPS O antigen (Amano et al. 1989, Shibuya et al. 1991), or that the serotype b antigen is an *A. actinomycetemcomitans* capsular material (McArthur et al. 1996). Although a crude LPS extraction method may not
separate the serotype-specific antigen from LPS (Amano et al. 1989, Shibuya et al. 1991), our present LPS extraction method seems to be a valid one, giving the same immunoblotting profile as phenol-extracted LPS from *A. actinomycetemcomitans* strain Y4 (serotype b).

Three of the six study subjects simultaneously harbored serotype c and nonserotypeable *A. actinomycetemcomitans* strains which comprised two-thirds of the 24 *A. actinomycetemcomitans* strains (Study II). This suggests that serotype c strains are more prone to alter their antigenicity, showing the same trend as the nonserotypeable *A. actinomycetemcomitans* strains (Study I). Similar changes in the LPS may occur among serotype b strains, although results for serotype b strains are based on only three strains from a single subject. Conversely, the LPS profiles of serotype a and nonserotypeable *A. actinomycetemcomitans* strains from the same subjects were similar in the immunoblots (Fig. 3A from original publication II). Reasons for the nonserotypeability of *A. actinomycetemcomitans* strains of serotype a-specific AP-PCR genotypes cannot be explained by the results with the present methods and need to be further investigated.

The serotypeable and nonserotypeable *A. actinomycetemcomitans* strains shared great similarity in outer membrane protein patterns both within one subject and between subjects (Fig. 1 from original publication II). This suggests homology of the major OMPs of *A. actinomycetemcomitans*, as has been shown by earlier studies; four to five major cell envelope proteins dominate in all serotypes studied (a, b, and c) of several *A. actinomycetemcomitans* strains (DiRienzo & Spieler 1983, Muller et al. 1990, Bolstad et al. 1990, van Steenbergen et al. 1994). Recently, however, differences in OMP patterns have been found between the rough and smooth colony morphologies within four *A. actinomycetemcomitans* strains: two proteins (43 and 20 kDa) are expressed by the rough variants exclusively (Haase et al. 1999). All of the clinical serotypeable and nonserotypeable *A. actinomycetemcomitans* strains studied here were of rough colony types and expressed these two proteins.

In some bacterial species, such as *P. aeruginosa* and *E. coli*, antimicrobial resistance may vary due to LPS or O antigen alterations (Leying et al. 1992, Tarr et al. 1998). The slight differences in antimicrobial susceptibility between serotypeable and nonserotypeable *A. actinomycetemcomitans* strains (Table 2 from original publication II) are not likely, however, to be due to LPS alterations. Differences in MICs were seen against only one of the six antimicrobial agents studied, in strains from two of the six subjects, and these were no more than two dilution steps away from each other.

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Characterization of nonoral *A. actinomycetemcomitans* strains (III)

The oral cavity is the ecological niche for *A. actinomycetemcomitans*, but the organism is occasionally isolated from infections at other than oral sites, as well. Presently it is unknown whether the nonoral environment favors any particular characteristics of *A. actinomycetemcomitans* or whether only certain *A. actinomycetemcomitans* strains cause nonoral infections. To study the possible differences between nonoral and oral *A. actinomycetemcomitans* strains, we determined serotypes and genotypes, antimicrobial susceptibilities, and leukotoxin gene promoter structures of 73 nonoral and oral *A. actinomycetemcomitans* strains. A total of 52 *A. actinomycetemcomitans* strains from 51 subjects with various nonoral infections (Table 1 from original publication III) were collected from distant geographic locations (See Material and Methods). Oral *A. actinomycetemcomitans* strains (*n* = 21) from our culture collection at the Institute of Dentistry, University of Helsinki, were obtained from 21 subjects. The oral strains matched the serotypes and genotypes of nonoral strains and were used as a control group for the leukotoxin gene determination and antimicrobial susceptibility testing.

**Serotype, genotype, and leukotoxin determinants of nonoral *A. actinomycetemcomitans***

Serotype and AP-PCR genotype distributions of the nonoral *A. actinomycetemcomitans* strains showed wide heterogeneity: all five known serotypes and two nonserotypeable strains were recovered (Table 2 from original publication III). The major serotypes were a, b, and c (16%, 41%, and 31% of subjects) and smaller proportions were of serotypes d and e, or nonserotypeable strains (8%, 2%, and 4%). One subject harbored two *A. actinomycetemcomitans* serotypes, b and c. The AP-PCR genotyping distinguished 10 different genotypes among the nonoral strains (Fig. 1 from original publication III), showing that nonoral *A. actinomycetemcomitans* infections may result from a variety of clones.

Serotype distribution of the present nonoral *A. actinomycetemcomitans* strains strongly resembles that of oral *A. actinomycetemcomitans* strains when all five serotypes are determined (Saarela et al. 1992, Yamamoto et al. 1997, Dogan et al. 1999a). *A. actinomycetemcomitans* serotype b, which is associated with periodontal disease (Zambon et al. 1983, Asikainen et al. 1991), was predominant (41% of subjects) in nonoral infections. The absence of virulent clonal types--characterized by a deletion in the leukotoxin gene promoter--among A.
*actinomycetemcomitans* strains from nonoral infections, corroborated that of oral strains of northern European and non-African origin, where this virulent clone has not been detected (Haubek et al. 1995, Asikainen et al. 1997). The present results agree with the idea of the oral cavity's being the ecological niche of *A. actinomycetemcomitans* and the likely origin of nonoral *A. actinomycetemcomitans* strains. However, the present findings on serotype distribution of nonoral *A. actinomycetemcomitans* strains differ from previous results (Zambon et al. 1988). The data of Zambon et al. (1988) suggest that serotype c predominates in nonoral infections: 22 (73%) of the 30 *A. actinomycetemcomitans* strains were of serotype c. The number of *A. actinomycetemcomitans* strains included in the present study and in that of Zambon et al. (1988) are still limited, which, together with the different serotyping methods, may account for the differences between the present and that study.

Four of the AP-PCR genotypes comprising as many as 22 (42%) of 52 nonoral strains had not been found in earlier studies (Asikainen et al. 1995, 1996, 1997, Study I, Dogan et al. 1999b). Since these works have mainly included oral *A. actinomycetemcomitans* strains from Finnish subjects, the present finding of previously undetectable AP-PCR genotypes is likely due to the widespread geographic origins of the present strains, but does not rule out the possible existence of specific *A. actinomycetemcomitans* clones in nonoral infections.

**Associations between *A. actinomycetemcomitans* clones and specific nonoral infections**

Generally, the characteristics of nonoral *A. actinomycetemcomitans* strains resembled those of the oral strains. However, some interesting correlations appeared between certain strains and the recovery site: blood or focal infections. Serotype b was more prevalent (49 versus 20%, Table 2 from original publication III) in blood samples from endocarditis and bacteremia patients than in focal infections which are likely less severe than blood infections. Additionally, *A. actinomycetemcomitans* AP-PCR genotype 3 occurred statistically significantly (*p*=0.020) more frequently in subjects with focal infections (33%) than in the blood samples (6%) (Table 2 from original publication III). These findings suggest that certain *A. actinomycetemcomitans* clones are of importance in nonoral infections. Due to serotype- or genotype-dependent factors, some *A. actinomycetemcomitans* strains may exhibit tropism for certain tissues, such as the endocardium. Previously, from a total of 15 distinguishable *A. actinomycetemcomitans* AP-PCR genotypes, this particular AP-PCR genotype 3 was the most frequent one (32%) in the oral cavities of periodontally healthy subjects (Asikainen et al. 1995). Further studies are, however, needed to determine whether certain characteristics
enable strains of this genotype to colonize healthy oral cavities or preferentially cause localized nonoral infections.

**Antimicrobial susceptibility patterns**

The antimicrobial agent susceptibility tests revealed no differences in MICs at which 50% (MIC$_{50}$) or 90% (MIC$_{90}$) of strains are inhibited between the nonoral and oral *A. actinomycetemcomitans* strains (Table 3 from original publication III). Amoxicillin, tetracycline, azithromycin, and trovafloxacin showed good activity against all *A. actinomycetemcomitans* strains, regardless of the recovery site. According to the NCCLS breakpoints suggested for *Haemophilus* spp. susceptibility interpretation (NCCLS 1998a, 1998b, and Table 3 from original publication III), it was observed that of all 73 *A. actinomycetemcomitans* strains studied, approximately 30% were resistant to benzylpenicillin and 5% to metronidazole. All the resistant strains were of serotypes a, b, or c (Table 4 from original publication III).

Our present results on the penicillin resistance of nonoral and oral *A. actinomycetemcomitans* strains largely corroborate those of other studies on the antimicrobial susceptibilities of oral *A. actinomycetemcomitans* strains (Slots et al. 1980b, Walker et al. 1985, Pajukanta et al. 1993, Avila-Campos et al. 1995, Madinier et al. 1999). *A. actinomycetemcomitans* strains do not produce penicillinase (Slots et al. 1980b), and therefore their resistance to benzylpenicillin does not seem to be beta-lactamase-mediated. Instead, it may be related to changes in penicillin-binding proteins, as has been observed with the penicillin resistance among strains of *H. influenzae* (Clairoux et al. 1992), a close phylogenetic relative of *A. actinomycetemcomitans*. Additionally, the poor permeability of the gram-negative outer membrane may account for the penicillin resistance of *A. actinomycetemcomitans*. Resistance to benzylpenicillin occurred among *A. actinomycetemcomitans* serotype b strains (62%), especially among two AP-PCR genotypes (2 and 12) of serotype b (Table 4 from original publication III). The same trend appeared both in nonoral and in oral *A. actinomycetemcomitans* strains. The benzylpenicillin resistance of *A. actinomycetemcomitans* is mainly of academic interest, since this antimicrobial agent is not the first choice in the treatment of chronic periodontal infections. However, the penicillin resistance of *A. actinomycetemcomitans* serotype b strains, suggesting specific properties such as new or altered penicillin-binding proteins, needs further study.
According to the NCCLS breakpoints for anaerobic bacteria (NCCLS 1998b) four (5%) of 73 A. actinomycetemcomitans strains, two nonoral and two oral strains, were resistant to metronidazole. Two of the resistant strains were of serotype b and two of serotype c, all four strains being of different AP-PCR genotypes. Several studies have shown that resistance to metronidazole occurs among oral A. actinomycetemcomitans strains (Slots et al. 1980b, Walker et al. 1985, Jousimies-Somer et al. 1988, Pajukanta et al. 1993, Avila-Campos et al. 1995, Madinier et al. 1999), which can be expected due to its aerotolerance. Amoxicillin, the currently recommended antimicrobial agent for use as endocarditis prophylaxis in dental procedures (Dajani et al. 1997), showed good activity against all present A. actinomycetemcomitans strains, and therefore can be anticipated to be effective as endocarditis prophylaxis for periodontitis patients harboring oral A. actinomycetemcomitans. The present results for amoxicillin corroborate other antimicrobial susceptibility studies for oral A. actinomycetemcomitans strains (Walker et al. 1985, Madinier et al. 1999). Additionally, trovafloxacin, a new quinolone which has not previously been tested against A. actinomycetemcomitans, showed high activity against the study strains. Trovafloxacin shows excellent activity against several microaerophilic bacterial species (Väisänen et al. 1997), but presently has been taken off the market, since it recently proved to be hepatotoxic in humans. However, the high activity of trovafloxacin shows that old quinolones, such as ciprofloxacin, norfloxacin, and ofloxacin, and new quinolones, such as levofloxacin, most likely will show good activity against A. actinomycetemcomitans.

**Heat shock response of A. actinomycetemcomitans and its effects on epithelial cells (IV)**

Heat shock protein (HSP) expression, which has been shown to contribute to the virulence of several organisms (Lathigra et al. 1991), may play a role in the pathogenic potential of A. actinomycetemcomitans. HSP expression has not previously been linked to A. actinomycetemcomitans infection. Therefore, 12 A. actinomycetemcomitans strains, representing all known serotypes from various oral and nonoral infections, were investigated to find differences in HSP expression among several A. actinomycetemcomitans strains. GroEL-like and DnaK-like HSPs were localized in cell fractions of A. actinomycetemcomitans, and the
effects of whole *A. actinomycetemcomitans* cells and protein-rich cell membrane fractions on periodontal ligament epithelial cells were studied.

**Localization and expression of heat shock proteins in A. actinomycetemcomitans**

GroEL- and DnaK-like proteins were detected both in heat-shocked and non-shocked *A. actinomycetemcomitans* cell fractions. HSP production in the absence of stress has previously been reported in *A. actinomycetemcomitans* (Goulhen et al. 1998), *E. coli* (Bukau et al. 1993), and *Haemophilus ducreyi* (Parsons et al. 1997). This is possible when HSPs, besides being protection against stressful conditions (Ellis 1996), are expressed in normal conditions during the cell cycle, development, and differentiation (Bukau & Horwich 1998). Methodological aspects may explain the minor differences observed between non-shocked and heat-shocked strains; the presently used heat-shocking time (2 h) may have been long enough for *A. actinomycetemcomitans* to induce an adaptation process instead of a mere stress response. It is also possible that the commonly used anaerobic and microaerophilic culture method itself is stressful for *A. actinomycetemcomitans*.

GroEL- and DnaK-like HSPs in the microbial cells are mainly intracellular proteins, but the localization may vary, depending on the organism. Since the HSP localization in several bacterial species is variable, the intracellular/cell surface localization may account for the different pathogenic characteristics of various bacteria. In all the *A. actinomycetemcomitans* strains, GroEL-like protein (HSP60) was found in the membranes, cytoplasm, and periplasm, whereas DnaK-like protein (HSP70) was present only in the cytoplasmic and periplasmic fractions (Fig. 1 from original publication IV). GroEL-like protein from two *A. actinomycetemcomitans* laboratory strains has been shown to be located in the soluble cell fractions and in the membrane fraction (Goulhen et al. 1998). This is similar to GroEL localization in *Helicobacter pylori* (Phadnis et al. 1996). DnaK-like protein has been reported to be localized intracellularly in *A. actinomycetemcomitans* (Goulhen et al. 1998), *Borrelia burgdorferi* (Scorpio et al. 1994), and *E. coli*, in which, additionally, a subpopulation of DnaK-like protein may become membrane-associated (Bukau et al. 1993). In some bacteria, expression of GroEL- and DnaK-like proteins is coupled. This is the case with *H. ducreyi*, where DnaK serves as a downregulating modifier of GroEL expression (Parsons et al. 1997). The relative amounts of GroEL- and DnaK-like protein expression in the *A. actinomycetemcomitans* strains could not, however, be determined in our study, because two distinct antibodies were used in the immunoblot analysis.
Some differences were observed in the GroEL- and DnaK-like protein expressions among the present *A. actinomycetemcomitans* strains. The GroEL expression in *A. actinomycetemcomitans* membrane fractions of different strains was defined as weak, moderate, or strong (Fig. 2 from original publication IV). In Fig. 3 from original publication IV, *A. actinomycetemcomitans* strains are arranged in order of increasing strength of GroEL expression in the membrane fraction. No correlation was observed between HSP expressions and the serotype or the origin (oral/nonoral, disease/health) of *A. actinomycetemcomitans* strains. One strain (CCUG 37418) from endocarditis expressed the membrane-associated GroEL-like protein strongly, whereas another strain (CCUG 37000R) from endocarditis expressed it weakly. Interestingly, a strain (IDH I31A) from one healthy oral cavity expressed GroEL-like protein more strongly than did two strains (JP2 and IDH 2765) from severe juvenile periodontitis.

**Relationship between membrane-associated GroEL expression and epithelial-cell proliferation**

The membrane fraction of *A. actinomycetemcomitans* is of special interest, because the HSP localization on the bacterial cell surface enables rapid HSP expression into the environment and makes possible the quick contact of HSP with human cells. At low concentrations (0.1-1.0 µg protein/ml) the membrane fraction of *A. actinomycetemcomitans* strains that expressed the GroEL-like protein moderately or strongly induced epithelial-cell proliferation stronger than did strains that expressed GroEL-like protein weakly (Fig. 3 from original publication IV). This suggests a correlation between membrane-associated expression of *A. actinomycetemcomitans* GroEL-like protein and epithelial-cell proliferation. The cell culture studies were made by using porcine periodontal ligament epithelial cells which had been isolated from epithelial rests of Malassez (Brunette et al. 1976) and represent a model for human junctional epithelium (Pan et al. 1995). Increased apical proliferation of epithelial cells may lead to deepening of periodontal pockets and thus a larger area for bacteria to grow. Therefore, GroEL-like HSP may account for the survival of *A. actinomycetemcomitans* in the periodontal pocket, and, by increasing epithelial proliferation, play a role in the virulence of *A. actinomycetemcomitans*. This fact supports the role of GroEL-like protein in the pathogenicity of clinical *A. actinomycetemcomitans* strains, as has been suggested earlier by Goulhen et al. (1998) and Kirby et al. (1995) on *A. actinomycetemcomitans* laboratory strains.
HSPs may play a role in the pathogenic potential of several microbial species. *Salmonella* serotype Typhimurium HSP66 is involved with the mucus-mediated interaction of this bacterium with its host’s intestinal mucosa (Ensgraber & Loos 1992). *H. pylori* HSP60 is associated with adhesion of *H. pylori* to human gastric epithelial cells (Yamaguchi et al. 1997), whereas *H. ducreyi* has diminished ability to adhere to human cells when GroEL-like protein expression is lowered (Parsons et al. 1997). *Legionella pneumophila* HSP60, found at elevated levels early in the infection, correlates with the virulence of this organism (Fernandez et al. 1996). Among other characteristics, differences in HSP expression between the present *A. actinomycetemcomitans* strains may contribute to variable host responses evoked by several clinical *A. actinomycetemcomitans* strains. At higher concentration (10 µg protein/ml), all the *A. actinomycetemcomitans* membrane fractions tested were cytotoxic to epithelial cells, suggesting that the number of *A. actinomycetemcomitans* cells in the periodontal pocket determines the effect--proliferation or death--on host cells. The relation of HSP expression to the course of an infection has not been completely established with any bacterial species; no previous studies are known to compare HSP expression between several strains from different clinical statuses. The present results suggest that GroEL-like HSP expression of *A. actinomycetemcomitans* plays a role in periodontal destruction, but further studies with mutant strains lacking GroEL-like HSP are needed to establish this relationship.

**Effects of *A. actinomycetemcomitans* whole cells on epithelial cells**

The effects of 12 *A. actinomycetemcomitans* strains on epithelial-cell proliferation or death were investigated by use of porcine periodontal ligament epithelial cell cultures in order to discover any differences in pathogenic potential between clinical *A. actinomycetemcomitans* strains. Various amounts of bacterial cell suspensions of optical density at 600 nm (OD<sub>600</sub>) = 0.5 were added to epithelial cell cultures. Thirty µl of bacterial cell suspensions comprising 10% of total volume of the culture medium was selected to be the concentration in the subsequent experiments. At this concentration, both increase in proliferation (Figure 1) and cytotoxic effects (Figure 2) were observed in the epithelial cells. Effects were variable between *A. actinomycetemcomitans* strains, with no apparent specificity to the serotype or origin of the strain. Proliferation and death of epithelial cells occur during the conversion of junctional epithelium to pocket epithelium, and both are important mechanisms in the formation of a periodontal pocket. Cell proliferation and death are functionally opposite effects, but they can be induced by the same effector molecules in a single cell type. For instance, increase in cell
proliferation and cell death have both been observed with interferon-γ in human malignant T-cells (Novelli et al. 1994), interleukin-1β in thymoma cells (Fratelli et al. 1995), *Mycobacterium tuberculosis* HSP10 in teratocarcinoma cells (Galli et al. 1996), and purified *A. actinomycetemcomitans* GroEL-like protein in epithelial cells (Goulhen et al. 1998).

The present results regarding effects on epithelial-cell proliferation and death cannot be directly compared between whole cells and cell fractions in the same *A. actinomycetemcomitans* strain because of the distinctive growing media used, agar plates for whole cells and broth for cell fractions. Although the growth atmosphere was anaerobic in both cases, the liquid medium may favor other characteristics of *A. actinomycetemcomitans* than on solid medium. The presently noted proliferative and cytotoxic effects of both non-shocked and heat-shocked *A. actinomycetemcomitans* strains on epithelial cells may not be explainable by expression of HSPs only, but are likely also due to other *A. actinomycetemcomitans* virulence factors, such as specific cytotoxins (Baehni et al. 1979, Tsai et al. 1979, Shenker et al. 1982, 1999), LPS (Kiley & Holt 1980), cell surface-associated materials (Meghji et al. 1992), or proteinase production (Suido et al. 1986, Uitto et al. 1988, Wang et al. 1999). However, based on epithelial cell culture studies with cell fractions of *A. actinomycetemcomitans* strains, GroEL-like heat shock protein appears to be one of the virulence determinants of *A. actinomycetemcomitans*. 
Figure 1. PLE cells were grown in the presence of whole *A. actinomycetemcomitans* cells and 10% serum. The number of viable PLE cells was measured with Cell Titer 96 Kit (Promega). Values are optical density at 570 nm (mean of 3 samples) reflecting number of viable cells. Mean value of cultures without added bacteria has been subtracted.

Figure 2. PLE cells were grown in the presence of whole *A. actinomycetemcomitans* cells in the absense of serum. Number of viable PLE cells was measured with Cell Titer 96 Kit (Promega). Values are optical density at 570 nm (mean of 3 samples). Results gave negative figures of viable PLE cells, showing a smaller amount of cells than the mean value of cell cultures without added bacteria. Negative figures reflecting PLE cell loss converted to positive figures reflecting number of dead cells.
SUMMARY AND CONCLUSIONS

The present studies investigated intraspecies differences in virulence-associated characteristics between *A. actinomycescomitans* strains originating from the oral cavity and nonoral infections. The focus was on a few specific characteristics such as nonserotypeability, nonoral origin, and the heat shock response.

Nonserotypeable oral *A. actinomycescomitans* strains were genotypically compared with serotypeable strains to study if they have a common origin. The nonserotypeable strains produced a heterogenic group of AP-PCR genotypes. Most of these AP-PCR genotypes were similar to those produced by serotypeable strains, and thus corresponded to a known serotype. This suggests that nonserotypeable strains originate from serotypeable ones. A total of 62% of all nonserotypeable strains produced an AP-PCR banding pattern corresponding to serotype c. This is a markedly higher portion than could have been expected based on the general serotype distribution among oral *A. actinomycescomitans* strains. The results suggest that the serotype c strains lose their ability to react with the serotype-specific antiserum more easily than do strains of the other serotypes. However, a small possibility of finding a new serotype among nonserotypeable *A. actinomycescomitans* strains still exists.

Outer membrane proteins (OMP) and lipopolysaccharide (LPS) profiles were compared between nonserotypeable and serotypeable oral *A. actinomycescomitans* strains from the same subject to find reasons for the altered antigenicity of nonserotypeable strains. The OMP patterns between serotypeable and nonserotypeable strains in each subject were similar. Differences in the LPS profiles were observed only in Western immunoblotting: nonserotypeable strains of serotype b- and c-specific AP-PCR genotypes lacked a clear LPS profile, a smear in the high-molecular-weight O-antigenic region of LPS clearly present in the serotypeable strains from the same subjects. These results suggest that altered antigenicity of the strains, especially of serotype b- and c-specific genotypes, is due to changes in the O-antigenic region of LPS.

Nonoral strains were compared with oral strains to see if certain characteristics of *A. actinomycescomitans* are more often found among strains from nonoral infections. Nonoral strains were serotypically and genotypically characterized, leukotoxin gene promoter structures were analyzed, and antimicrobial susceptibilities were studied. Generally, these characteristics were very similar between the nonoral strains and a control group of oral strains. Serotype b, in
particular, predominated in nonoral infections. Serotype b was most frequently found from bacteremia or endocarditis samples, and a certain AP-PCR genotype correlated with focal infections. These results support the importance of certain clones in nonoral A. actinomycetemcomitans infections. That four previously unknown AP-PCR genotypes were found may have been due to the non-Finnish origin of the strains. Additionally, elevated resistance was observed of both oral and nonoral A. actinomycetemcomitans serotype b strains to benzylpenicillin.

The heat shock protein (HSP) production in A. actinomycetemcomitans strains and its contribution to the ability to induce epithelial-cell proliferation or death were studied to seek differences between 12 strains of various clinical origins. In all strains studied, GroEL-like protein (HSP60) was located intracellularly and on the cell surface, whereas DnaK-like protein (HSP70) was found only intracellularly. HSP expression varied between strains, but did not correlate with the serotype or the origin of these strains. All strains, both heat-shocked and non-shocked, had proliferation-promoting or cytotoxic effects on periodontal ligament epithelial cells. The strength of GroEL-like protein expression in the membrane fraction of A. actinomycetemcomitans correlated positively with epithelial-cell proliferation; strains that expressed the GroEL-like protein moderately or strongly induced epithelial-cell proliferation more strongly than did strains that expressed the protein weakly. This suggests that GroEL-like protein plays a role in the virulence of A. actinomycetemcomitans by increasing epithelial proliferation.

Taken together, the present studies on certain virulence-associated characteristics of A. actinomycetemcomitans extend knowledge of the pathogen in several ways. The studies provide information on the genetic heterogeneity of serologically nontypeable strains and present possible reasons for A. actinomycetemcomitans nonserotypeability. The studies additionally characterize this oral pathogen in nonoral infections and show a positive correlation between A. actinomycetemcomitans heat shock protein expression and epithelial-cell proliferation.

Based on the present studies, the main conclusions are that:
- Nonserotypeable oral A. actinomycetemcomitans strains originate from serotypeable ones, especially from serotype c, and the possibility of finding additional serotypes of the species is small.
- *A. actinomycetemcomitans* serotype c strains lose their ability to react with the serotype-specific antiserum more easily than do strains of the other serotypes.
- The altered antigenicity and nonserotypeability of certain *A. actinomycetemcomitans* strains, especially strains of serotype b- and c-specific genotypes, seems to be due to changes in the O-antigenic region of LPS.
- The serotype and genotype characterization, leukotoxin gene determinants, and antimicrobial susceptibility patterns of nonoral *A. actinomycetemcomitans* strains greatly resemble those of oral strains and thus suggest that the origin of nonoral *A. actinomycetemcomitans* is the oral cavity.
- The predominance of serotype b strains in nonoral *A. actinomycetemcomitans* infections, and relationships between certain serotypes or genotypes and specific infections support the importance of certain *A. actinomycetemcomitans* clones in nonoral infections.
- Oral and nonoral *A. actinomycetemcomitans* serotype b strains are resistant to benzylpenicillin more often than are strains of the other serotypes.
- Heat-shocked as well as non-shocked *A. actinomycetemcomitans* strains of all known serotypes may exert both cytotoxic and hyperproliferative effects on epithelial cells.
- A positive correlation exists between membrane-associated expression of *A. actinomycetemcomitans* GroEL-like heat shock protein and epithelial-cell proliferation.
- GroEL-like heat shock protein may play a role in the virulence of *A. actinomycetemcomitans* by increasing epithelial-cell proliferation.
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ACKNOWLEDGMENTS

This study was carried out at the Department of Periodontology, Institute of Dentistry, University of Helsinki, during the years 1996-1999. In addition, parts of this study have been carried out at the Anaerobe Reference Laboratory, National Public Health Institute, Helsinki; at the Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, Canada; and at the Groupe de Recherche en Écologie Buccale, Université Laval, Québec, Canada.

My warmest thanks go to my supervisor Docent Sirkka Asikainen, DDS, PhD, for showing me the way into the wonderful world of science. I express my deepest gratitude to her for guiding and encouraging me during this study. With her vast knowledge, enthusiasm, and manner of scientific thinking, she has given me an admirable role-model as a researcher.

I extend my warm and sincere thanks to my second supervisor Professor Veli-Jukka Uitto, DDS, PhD. I have been privileged to benefit from his comprehensive expertise. His personable support and guidance, along with the invaluable training for the scientific competitions, have been of irreplaceable help.

I am profoundly grateful to the official referees of this thesis, Docent Anja Siitonen, PhD, and Docent Matti Viljanen, MD, PhD, for their constructive criticism and valuable suggestions.

I sincerely thank my co-authors Docent Hannele Jousimies-Somer, PhD, Docent Maria Saarela, PhD, Dr. Petteri Carlson, MD, PhD, Professor Casey Chen, DDS, PhD, Professor Satu Alaluusua, DDS, PhD, and Professor Paula Fives-Taylor, PhD, for their collaboration and sharing their knowledge with me. Professor Pirjo Mäkelä, MD, PhD, is especially recognized for her advice concerning Study II.

A warm-hearted Merci goes to Québec to Professor Denis Mayrand, PhD, Professor Daniel Grenier, PhD, Florence Goulhen, MSc, and Pascale Plamondon, MSc, for their kind help and pleasant collaboration.

I wish to thank Dr. Lianxuan Zhang, MD, for his help in Study IV, and Jaana Mättö, PhD, Heini Torkko, MSc, and Kirsti Kari, MSc, for their help with the laboratory work. I also thank Ms. Annikki Sirén for technical assistance. I express my thanks to Carol Norris, PhD, for revising the language of this thesis.

I warmly thank my mother and father for their support and for always encouraging me in everything I decide to do. Special thanks go to my sister Heidi for helping me to keep myself in one piece in the changeable periods of this study.

Finally, I thank everyone for good chemistry and bad chemistry; you cannot create and develop without both.

This study was financially supported by the University of Helsinki (Scholarship for Young Researchers), the Finnish Dental Society, and the Finnish Women Dentists’ Association.

Helsinki, January 2000

Susanna Paju