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Clinical Periodontology and Implant Dentistry

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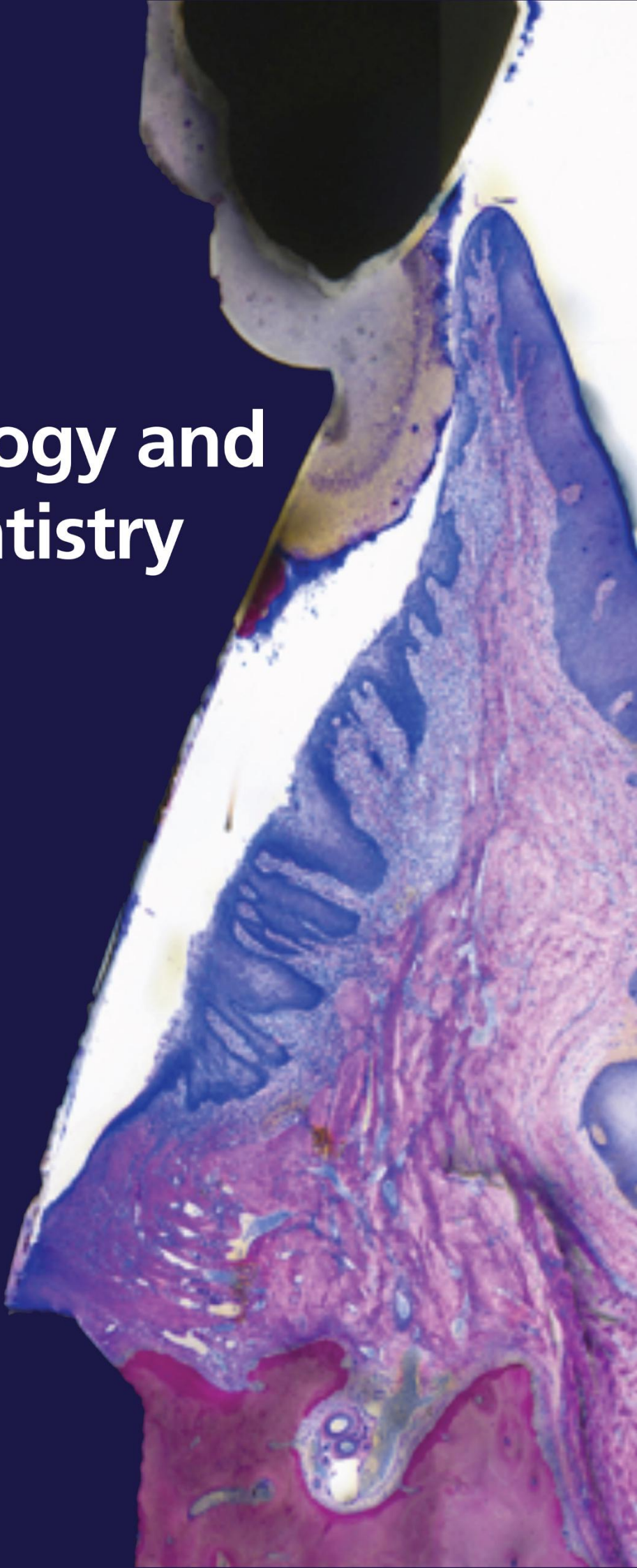

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isms that were capable of forming large amounts of plaque *in vivo* and *in vitro* should be considered as prime suspects in the etiology of periodontal diseases. Human isolates of *Actinomyces* species were shown to have this ability *in vitro* and led to plaque formation and periodontal destruction in animal model systems. These findings reinforced the notion that organisms that formed abundant plaque were responsible for destructive periodontal disease. Unfortunately, later research findings revealed major discrepancies in this hypothesis.

Return to specifi city in microbial etiology of periodontal diseases

In the 1960s, interest in the specifi c microbial etiology of periodontal disease was rekindled by two groups of experiments. The fi rst demonstrated that peri-odontal disease could be transmitted in the hamster from animals with periodontal disease to animals without periodontal disease by caging them together. Swabs of plaque or feces from diseased animals were effective in transmitting the disease to animals free of disease. It was demonstrated that a pure culture of a Gram-positive pleomorphic rod that later became known as *Actinomyces viscosus* was capable of causing destructive periodontal disease in animals free of disease. Other species isolated from the plaque of hamsters with periodontal disease did not have this capability.

At about the same time, it was demonstrated that spirochetes with a unique ultrastructural morphology could be detected in practically pure culture in the connective tissue underlying lesions of ANUG and within the adjacent epithelium. Control tissue taken from healthy individuals and individuals with other forms of disease did not exhibit a similar tissue invasion. To date, the spirochete associated with ANUG has not been cultivated.

Such fi ndings suggested that there might be more specifi city to the microbial etiology of periodontal disease than had been accepted for the previous 4 decades. However, the emphasis of clinicians in the 1960s was on the mechanical control of plaque accu-mulation. This approach was consistent with the pre-vailing concept that periodontal disease was due to a non-specifi c accumulation of bacteria on tooth sur-faces. This concept is very much in evidence today and still serves as the basis of preventive techniques in most dental practices. It is also clear that non-specifi c plaque control is not able to effectively prevent all forms of periodontal disease.

The transmissibility studies stimulated a new concept of periodontal diseases. The organisms which were responsible for the periodontal destruction observed in the hamster clearly differed from other organisms by their ability to form large amounts of bacterial plaque both in the hamster and in *in vitro* test systems. A concept emerged that microorgan-

Changing concepts of the microbial etiology of periodontal diseases

By the end of the 1960s it was generally accepted that dental plaque was in some way associated with human periodontal disease. It was believed that the presence of bacterial plaque initiated a series of as yet undefined events that led to the destruction of the periodontium. The composition of plaque was thought to be relatively similar from patient to patient and from site to site within patients. Variability was recognized, but the true extent of differences in bacterial composition was not appreciated. It was thought that the major event triggering destructive periodontal disease was an increase in mass of bacterial plaque, possibly accompanied by a diminution of host resistance. Indeed, in the mid 1960s the classic studies of Loe *et al*. (1965, 1967) and Theilade *et al*. (1966) convincingly demonstrated that plaque accumulation directly preceded and initiated gingivitis. Many investigators believed that gingivitis was harmful, and led to the eventual destruction of the periodontal tissues, probably by host-mediated events.

Yet, certain discrepancies continued to baffle clinicians and research workers alike. If all plaques were more or less alike and induced a particular tissue response in the host, why was periodontal destruction localized, taking place adjacent to one tooth but not another? If plaque mass was a prime trigger for periodontal destruction, why did certain subjects accumulate much plaque, frequently accompanied by gingivitis, but fail, even after many years to develop destruction of the supporting structures? On the other hand, why did some individuals with little detectable plaque or clinical inflammation develop rapid periodontal destruction? If inflammation was the main mediator of tissue destruction, why were so many teeth retained in the presence of continual gingivitis? One explanation may have been that there were inconsistencies in the host response, or disease required the superimposition of local factors such as trauma from occlusion, overhanging fillings etc. Other explanations can be derived from extensive studies of the microbiota adjacent to periodontal tissues.

The recognition of differences in the composition of bacterial plaque from subject to subject and site to site within subjects led to a series of investigations.

Some studies attempted to determine whether specific microorganisms were found in lesion sites as compared to healthy sites. Others studies sought differences in the microorganisms in subgingival plaque samples taken from subjects with clinically different forms of periodontal disease or periodontal health. Newman *et al*. (1976, 1977) and Slots (1976) demonstrated that the microbial composition of subgingival plaque taken from diseased sites differed substantially from the samples taken from healthy sites in subjects with localized aggressive periodontitis (LAP). Tanner *et al*. (1979) and Slots (1977) demonstrated that the microbiota recovered from lesion sites from subjects with chronic periodontitis differed from the microbiota from healthy sites in the same subjects and also from lesion sites in LAP subjects. These studies, along with the demonstration that subjects with LAP could be treated successfully with local debridement and systemic antibiotics, provided the initial impetus to perform larger-scale studies attempting to relate specific microorganisms to the etiology of different periodontal diseases.

Current suspected pathogens of destructive periodontal diseases

Criteria for defining periodontal pathogens

For more than a century, the classical "Koch's postulates" have been used to define a causal relationship between an infectious agent and a disease. These postulates were: (1) the agent must be isolated from every case of the disease, (2) it must not be recovered from cases of other forms of disease or non-pathogenically, and (3) after isolation and repeated growth in pure culture, the pathogen must induce disease in experimental animals (Carter 1987). The criteria for defining pathogens of destructive periodontal diseases initially were based on Koch's postulates but have been amended and extended in recent years. These criteria include association, elimination, host response, virulence factors, animal studies, and risk assessment. The discrimination of a pathogen from a non-pathogenic species is not based on a single criterion but rather on a "weight of evidence" evaluation.

The criterion of association is really the same as Koch's first two postulates; i.e. the species should be found more frequently and in higher numbers in cases of the infection than in individuals without overt disease or with different forms of disease. However, periodontal microbiologists do not expect to find the pathogen in "all cases of the disease" because they currently cannot distinguish "all cases of a given disease". The criterion of elimination is based on the concept that elimination of a species should be accompanied by a parallel remission of disease. If a species is eliminated by treatment and the disease progresses, or if the level of a species remains high or increases in a site and the disease stops, doubt would be cast on that species' role in pathogenesis. This criterion (like all of the others) has certain problems in that therapy rarely (if ever) eliminates or suppresses only one species at a time. The criterion of host response, particularly the immunological response, appears to be of value in defining periodontal pathogens. If a species (or its antigens) gains access to underlying periodontal tissues and causes damage, it seems likely that the host will produce antibodies or a cellular immune response that is directed specifically to that species. Thus, the host response could act as a pointer to the pathogen(s). Biochemical determinants (virulence factors) may also provide valuable clues to pathogenicity. Potentially damaging metabolites produced, or properties possessed, by certain species may be suggestive that those species could play a role in the disease process.

Animal model systems provide suggestive evidence that a microbial species may play a role in human disease. Particularly noteworthy are studies of experimentally induced disease in dogs or monkeys, which can be manipulated to favor selection of single or subsets of species that may or may not induce pathology. These models usually suggest a possible etiologic role of a species indigenous to the test animal that may have analogues in the human subgingival microbiota. Finally, technological developments, such as checkerboard DNA–DNA hybridization (Fig. 9-1) and polymerase chain reaction (PCR), now permit assessment of specific microorganisms in large numbers of subgingival plaque samples. This allows prospective studies to be performed in which the risk of periodontal disease progression conferred by the presence of an organism at given levels may be assessed.

Periodontal pathogens

The World Workshop in Periodontology (Consensus Report, 1996) designated *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* as periodontal pathogens. Tables 9-1, 9-2, and 9-3 summarize some of the data that indicate an etiologic role of these species in periodontal diseases, categorized according to the criteria defined above. The summary is by no means exhaustive but does indicate that a growing literature suggests some reasonable candidates as etiologic agents of destructive periodontal diseases.

Aggregatibacter (formerly *Actinobacillus*) *actinomycetemcomitans*

One of the clearest associations between a suspected pathogen and destructive periodontal disease is provided by *A. actinomycetemcomitans.* This species has recently been renamed *Aggregatibacter actinomycetemcomitans* from its former name of *Actinobacillus actinomycetemcomitans* (Norskov-Lauritsen & Kilian 2006). *A. actinomycetemcomitans* is a small, non-motile,

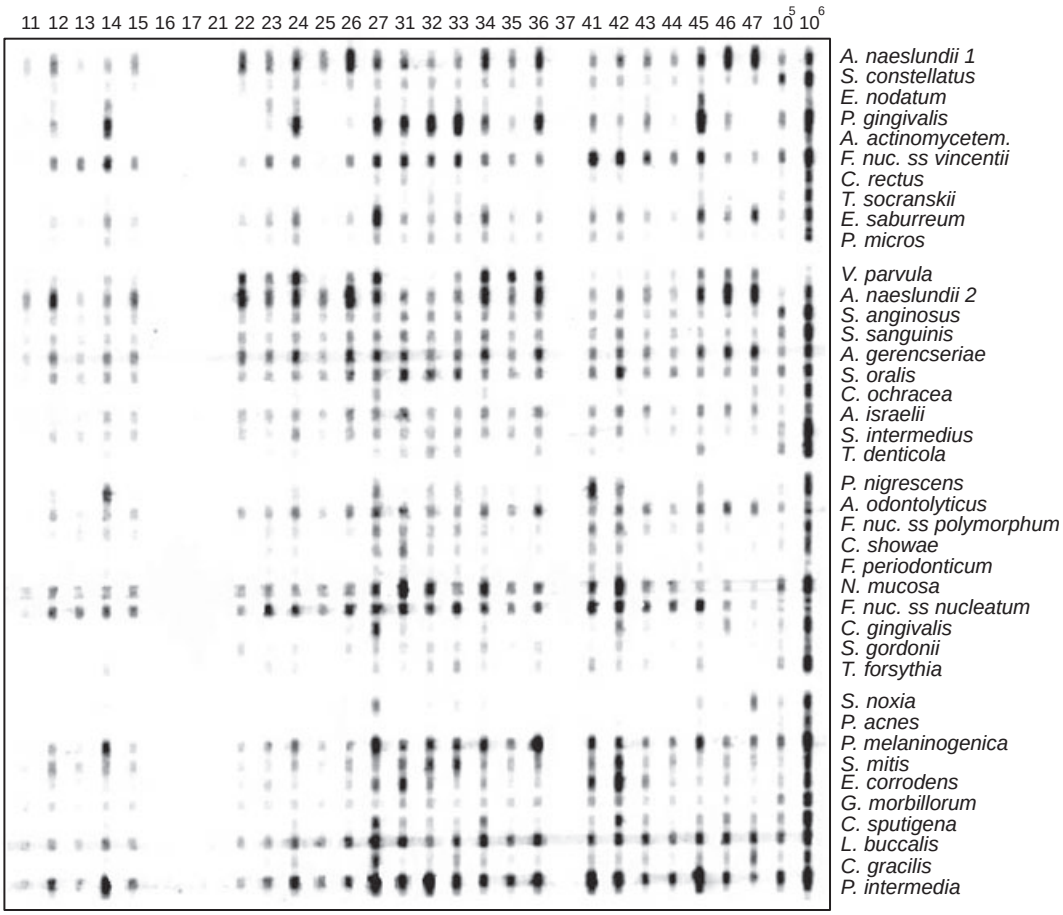

Fig. 9-1 Example of checkerboard DNA–DNA hybridization being used to detect 40 bacterial species in 28 subgingival plaque samples from a single patient. The vertical lanes are the plaque samples numbered from 11 (upper right central incisor) to 47 (lower right second molar). In this subject, teeth 16, 17, 21, and 37 were missing. The two vertical lanes on the right are standards containing either 10^5 or 10^6 cells of each test species. The horizontal lanes contained the indicated DNA probes in hybridization buffer. A signal at the intersection of the vertical and horizontal lanes indicates the presence of a species. The intensity of the signal is related to the number of organisms of that species in the sample. In brief, samples of plaque were placed into individual Eppendorf tubes and the DNA released from the microorganisms by boiling in NaOH. After neutralization, the released DNA was transferred to the surface of a nylon membrane using the 30 channels of a Minislot device (Immunetics, Cambridge, MA). The DNA was fixed to the membrane by UV light and baking and placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at right angles to the 45 channels of the Miniblotter device. Whole genomic DNA probes labelled with digoxigenin were placed in hybridization buffer into 40 of the lanes and hybridized overnight. After stringency washing, the signals were detected using phosphatase-conjugated antibody to digoxigenin and chemifluorescence substrates. Signals were compared to the standards using a Storm Fluorimager and converted to counts.

Table 9-1 Summary of some of the types of data that suggest that *Aggregatibacter actinomycetemcomitans* may be an etiologic agent of destructive periodontal diseases (for literature citations see text and Haffajee & Socransky 1994)

Factor	Data
Association	Elevated in lesions of localized juvenile periodontitis (LJP), prepubertal or adolescent periodontal disease
	Lower in health, gingivitis and edentulous subjects or sites
	Elevated in some adult periodontitis lesions
	Elevated in active lesions of juvenile periodontitis
	Detected in prospective studies
	Detected in apical ares of pocket or in tissues from LJP lesions
Elimination	Elimination or suppression resulted in successful therapy
	Recurrent lesions harbored the species
Host response	Elevated antibody in serum or saliva of LJP patients
	Elevated antibody in serum or saliva of chronic periodontitis patients
	Elevated local antibody in LJP sites
Virulence factors	Leukotoxin; collagenase; endotoxin; epitheliotoxin; fibroblast inhibitory factor; bone resorption inducing factor; induction of cytokine production from macrophages; modification of neutrophil function; degradation of immunoglobulins; cytolethal distending toxin (Cdt); induces apoptotic cell death
	Invades epithelial and vascular endothelial cells *in vitro* and buccal epithelial cells *in vivo*
Animal studies	Induced disease in gnotobiotic rats
	Subcutaneous abscesses in mice

Table 9-2 Summary of some of the types of data that suggest that *Porphyromonas gingivalis* may be an etiologic agent of destructive periodontal diseases (for literature citations see text and Haffajee & Socransky 1994)

Factor	Data
Association	Elevated in lesions of periodontitis Lower in sites of health, gingivitis and edentulous subjects Elevated in actively progressing lesions Elevated in subjects exhibiting periodontal disease progression Detected in cells or tissues of periodontal lesions Presence indicates increased risk for alveolar bone loss and attachment level loss
Elimination	Elimination resulted in successful therapy Recurrent lesions harbored the species Successful treatment lowered level and/or avidity of antibody
Host response	Elevated antibody in serum or saliva in subjects with various forms of periodontitis Altered local antibody in periodontitis
Virulence factors	Collagenase; endotoxin; proteolytic trypsin-like activity; fibrinolysin; hemolysin; other proteases including gingipain; phospholipase A; degrades immunoglobulin; fibroblast inhibitory factor; H_2S; NH_3; fatty acids; factors that adversely affect PMNs; capsular polysaccharide; bone resorption inducing factor; induction of cytokine production from various host cells; generates chemotactic activities; inhibits migration of PMNs across epithelial barriers; Invades epithelial cells *in vitro*
Animal studies	Important in experimental pure or mixed subcutaneous infections Induced disease in gnotobiotic rats Studies in sheep, monkeys and dogs Immunization diminished disease in experimental animals

Table 9-3 Summary of some of the types of data that suggest that *Tannerella forsythia* may be an etiologic agent of destructive periodontal diseases (for literature citations see text and Haffajee & Socransky 1994)

Factor	Data
Association	Elevated in lesions of periodontitis Lower in sites of health or gingivitis Elevated in actively progressing lesions Elevated in periodontal abscesses Increased in subjects with refractory periodontitis Detected in epithelial cells of periodontal pockets Presence indicates increased risk for alveolar bone loss, tooth and attachment level loss
Elimination	Elimination resulted in successful therapy Recurrent lesions harbored the species Reduced in successfully treated peri-implantitis
Host response	Elevated antibody in serum of periodontitis subjects and very high in a subset of subjects with refractory periodontitis
Virulence factors	Endotoxin; fatty acid and methylglyoxal production; induces apoptotic cell death; cytokine production from various host cells; invades epithelial cells *in vitro* and *in vivo*
Animal studies	Increased levels in ligature-induced periodontitis and peri-implantitis in dogs Induced disease in gnotobiotic rats

Gram-negative, saccharolytic, capnophilic, round-ended rod that forms small, convex colonies with a "star-shaped" center when grown on blood agar plates (Fig. 9-2). This species was first recognized as a possible periodontal pathogen by its increased frequency of detection and higher numbers in lesions of localized aggressive periodontitis (Newman *et al*. 1976; Slots 1976; Newman & Socransky 1977; Slots *et al*. 1980; Mandell & Socransky 1981; Zambon *et al*. 1983a; Chung *et al*. 1989) when compared with numbers in plaque samples from other clinical conditions including periodontitis, gingivitis, and health. Soon thereafter, it was demonstrated that the majority of subjects with localized aggressive periodontitis (LAP) had an enormously elevated serum antibody response to this species (Genco *et al*. 1980; Listgarten *et al*. 1981; Tsai *et al*. 1981; Altman *et al*. 1982; Ebersole *et al*. 1982, 1987) and that there was local synthesis of antibody to this species (Schonfeld & Kagan 1982; Ebersole *et al*. 1985; Smith *et al*. 1985; Tew *et al*. 1985a). When subjects with this form of disease were treated successfully, the species was eliminated or lowered in level; treatment failures were associated with failure to lower the numbers of the species in treated sites (Slots & Rosling 1983; Haffajee *et al*. 1984; Christersson *et al*. 1985; Kornman & Robertson 1985;

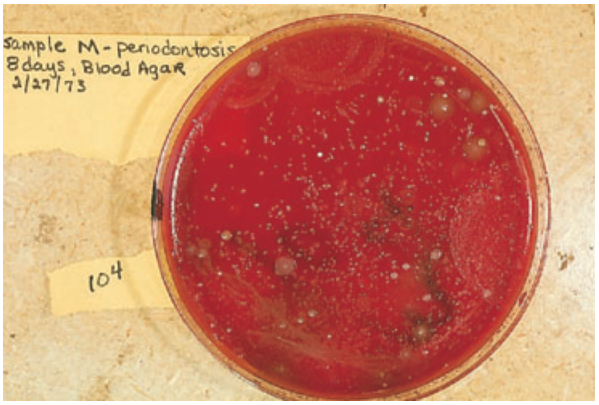

Fig. 9-2 Photograph of a primary isolation plate of a subgingival plaque sample from a diseased site in a subject with LAP. A dilution of the plaque sample was grown for 7 days at 35°C on an enriched blood agar plate in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. The majority of the small, round, convex colonies on this plate were isolates of *Aggregatibacter actinomycetemcomitans*.

Mandell *et al*. 1986; Preus 1988; Shiloah *et al*. 1998; Tinoco *et al*. 1998). The species produced a number of potentially damaging metabolites, including a leukotoxin (Baehni *et al*. 1979) and a cytolethal distending toxin (Saiki *et al*. 2001; Shenker *et al*. 2001), and induced disease in experimental animals (Irving *et al*. 1978). *A. actinomycetemcomitans* has been shown, *in vitro*, to have the ability to invade cultured human gingival epithelial cells (Blix *et al*. 1992; Sreenivasan *et al*. 1993), human vascular endothelial cells (Schenkein *et al*. 2000) and buccal epithelial cells *in vivo* (Rudney *et al*. 2001). Further, studies have shown that *A. actinomycetemcomitans* induced apoptotic cell death (Arakawa *et al*. 2000; Kato *et al*. 2000).

Perhaps the strongest association data came from studies of "active lesions" in which the species was elevated in actively progressing periodontal lesions when compared with non-progressing sites (Haffajee *et al*. 1984; Mandell 1984; Mandell *et al*. 1987; Haubek *et al*. 2004) and in prospective studies of as yet undiseased siblings of LAP subjects (DiRienzo *et al*. 1994). *A. actinomycetemcomitans* was also elevated in studies of disease progression in young Indonesian subjects (Timmerman *et al*. 2001). Collectively, the data suggest that *A. actinomycetemcomitans* is a probable pathogen of LAP. However, this should not be interpreted as meaning that it is the sole cause of this clinical condition, since a subset of subjects with LAP did not exhibit this species in samples of their subgingival plaque and had no elevated antibody response to the species (Loesche *et al*. 1985; Moore 1987).

The possibility that a subset of *A. actinomycetemcomitans* clonal types was primarily responsible for LAP was raised in studies at the University of Pennsylvania. Strains of *A. actinomycetemcomitans* were isolated from members of 18 families with at least one member with active LAP as well as from from 32 control subjects. Restriction fragment length polymorphisms (RFLP) indicated 13 distinct RFLP groups of *A. actinomycetemcomitans* (DiRienzo & McKay 1994). Isolates from LAP subjects fell into predominantly RFLP pattern II, while RFLP patterns XIII and XIV were seen exclusively in isolates from periodontally healthy subjects. Further, disease progression was related strongly to the presence of RFLP group II (DiRienzo *et al*. 1994).

Haubek *et al*. (1996) demonstrated that strains of *A. actinomycetemcomitans* isolated from families, initially of African origin living in geographically different areas, were characterized by a 530 base pair deletion in the leukotoxin gene operon leading to a significantly increased production of leukotoxin. They speculated that this virulent clonal type may account for an increased prevalence of LAP in African Americans and other individuals of African descent. A key isolate of this clonal type, strain JP2, was first isolated from an 8-year-old African American child with prepubertal periodontitis (Tsai *et al*. 1979; Kilian *et al*. 2006). There was a strong association between the presence of the JP2 clonal type of *A. actinomycetemcomitans* and early onset periodontitis in Moroccan school children, but no association between the presence of *A. actinomycetemcomitans* without the 530 bp deletion and early onset periodontitis (Haubek *et al*. 2001). Further, the odds ratio for disease progression in a subject in this population infected with the JP2 clone was 14.5 (Haubek *et al.* 2004). These observations were corroborated in a Brazilian population, where highly leukotoxic strains of *A. actinomycetemcomitans* were more prevalent in aggressive periodontitis than in chronic periodontitis (Cortelli *et al*. 2005). This deletion in the leukotoxin operon was not detected in any strains of *A. actinomycetemcomitans* isolated from adult Chinese subjects (Mombelli *et al.* 1999; Tan *et al*. 2001) or Asian subjects in the United States (Contreras *et al*. 2000). Subjects harboring *A. actinomycetemcomitans* with the 530 bp deletion were 22.5 times more likely to convert to LAP than subjects who had *A. actinomycetemcomitans* variants containing the full-length leukotoxin promoter region (Bueno *et al*. 1998). Interestingly, strains of *A. actinomycetemcomitans* with the RFLP type II pattern described by DiRienzo & McKay (1994) that were found frequently in LAP subjects included strains of the JP2 clonal type (Kilian *et al*. 2006). The above data suggest that *A. actinomycetemcomitans* is a major pathogen of LAP and that the JP2 clonal type is a key pathogen in certain human populations.

A. actinomycetemcomitans has also been implicated in adult forms of destructive periodontal disease, but its role is less clear. The species has been isolated from chronic periodontitis lesions, but less frequently and in lower numbers than from lesions in LAP subjects (Rodenburg *et al*. 1990; Slots *et al*. 1990a). In addition, its numbers in plaque samples from lesions in adults were often not as high as those observed for other suspected pathogens in the same plaque samples. There appear to be at least six serotypes of

A. actinomycetemcomitans (a, b, c, d, e, and f) and these serotypes appear to be clonal in nature (Kilian *et al*. 2006). The most frequently isolated serotype of *A. actinomycetemcomitans* from lesions of LAP in American subjects was serotype b (Zambon *et al*. 1983b), whereas serotype a was more commonly detected in samples from chronic periodontitis subjects (Zambon *et al*. 1983a). This finding was corroborated indirectly by examination of serum antibody levels to the two serotypes. Most elevated responses to *A. actinomycetemcomitans* in LAP subjects were to serotype b while elevated responses to serotype a were more common in adult subjects with chronic periodontitis (Listgarten *et al*. 1981). Some subjects in each group exhibited elevated serum antibody responses to both serotypes. In Finnish subjects, serotypes a and b were more frequently isolated from subjects with periodontal disease and serotype c from periodontally healthy subjects (Asikainen *et al*. 1991). However, this pattern of serotype distribution was not observed in Korea (Chung *et al*. 1989) or Japan (Saito *et al*. 1993; Yoshida *et al*. 2003), where *A. actinomycetemcomitans* serotype c was frequently observed in plaque samples from sites of periodontal pathology. Serotypes d, e, and f, have been recognized more recently (Dogan *et al*. 1999; Mombelli *et al*. 1999) and are found less frequently than serotypes a, b and c. For example, serotypes d, e or f were not detected in a Brazilian population (Teixeira *et al*. 2006) and serotypes d or e were not found in Taiwanese subjects <35 years of age with different forms of periodontitis (Yang *et al*. 2004a).

Antibody data and data from the treatment of *A. actinomycetemcomitans* infected patients with adult or refractory periodontitis provide the most convincing evidence of a possible etiologic role of *A. actinomycetemcomitans* in adult forms of periodontal disease. Thirty-six of 56 adults with destructive periodontal disease examined at multiple time periods at the Forsyth Institute exhibited an elevated serum antibody response to *A. actinomycetemcomitans* serotypes a and/or b. Elevated responses to other suspected periodontal pathogens were far less common. Van Winkelhoff *et al*. (1992) treated 50 adult subjects with "severe generalized periodontitis" and 40 subjects with refractory periodontitis who were culture positive for *A. actinomycetemcomitans* using mechanical debridement and systemically administered amoxicillin and metronidazole. Only one of 90 subjects was culture positive for *A. actinomycetemcomitans* 3–9 months post-therapy (van Winkelhoff *et al*. 1992) and one of 48 subjects was culture positive 2 years post-therapy (Pavicic *et al*. 1994). There was a significant gain in attachment level and decrease in probing pocket depth in virtually all patients after therapy.

It is suspected that *A. actinomycetemcomitans* initially colonizes the oral cavity by attachment to the surfaces of the oral epithelium (Fine *et al*. 2006). There is a specific protein adhesin, Aae, that binds to a carbohydrate receptor on buccal epithelial cells of humans and Old World monkeys. It is thought that *A. actinomycetemcomitans* moves from the buccal epithelial cells to the supragingival plaque, possibly binding to the tooth by fimbriae comprised of repeating subunits of a 6.5 kDa protein, Flp 1. The fimbiriae, along with an extracellular carbohydrate polymer, PGA, mediate attachment to hard surfaces (Fine *et al*. 2006). Alternatively, *A. actinomycetemcomitans* may attach to other colonizing bacterial species by coaggregation (Kolenbrander 2000). At some point these organisms may move from the supragingival to the subgingival environment. From this vantage point, they may then attach to and invade the epithelial lining of the periodontal pocket and possibly penetrate the underlying connective tissues (Rudney *et al*. 2001). *A. actinomycetemcomitans* has been shown to be present in the intima of vessel walls (Marques de Silva *et al*. 2005) and has been cultured from atheromatous plaques (Kozarov *et al*. 2005). Finally, *A. actinomycetemcomitans* may leave the oral cavity and cause or contribute to endocarditis, since it has been frequently found in lesions of this condition (Paturel *et al*. 2004).

Porphyromonas gingivalis

P. gingivalis is a second consensus periodontal pathogen that continues to be intensely investigated. Isolates of this species are Gram-negative, anaerobic, non-motile, asaccharolytic rods that usually exhibit coccal to short rod morphologies. *P. gingivalis* is a member of the much investigated "black-pigmented *Bacteroides*" group (Fig. 9-3). Organisms of this group form brown to black colonies (Oliver & Wherry 1921) on blood agar plates and were initially grouped into a single species, *B. melaninogenicus* (*Bacterium melaninogenicum*; Burdon 1928). The black-pigmented *Bacteroides* have a long history of association with periodontal diseases since the early efforts of Burdon (1928) through the mixed infection studies of Macdonald *et al*. (1960) to the current intense interest.

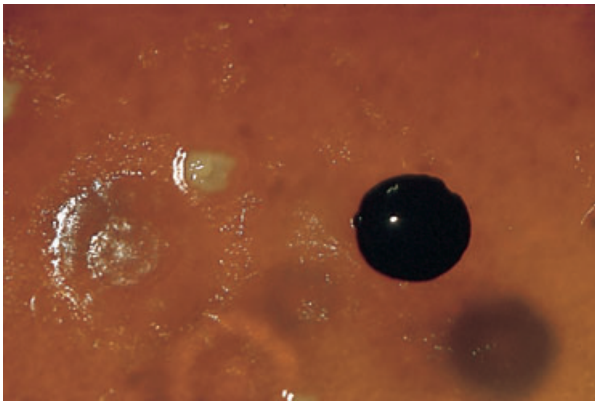

Fig. 9-3 Photograph of part of a primary isolation plate of a subgingival plaque sample from a subject with chronic periodontitis. The medium and growth conditions were as described in Fig. 9-2. The black-pigmented colony was an isolate of *Porphyromonas gingivalis*.

In the late 1970s, it was recognized that the black-pigmented *Bacteroides* contained species that were asaccharolytic (eventually *P. gingivalis*), and either had an intermediate level of carbohydrate fermentation (which eventually led to a group of species including *Prevotella intermedia*) or were highly saccharolytic (leading to the group that includes *Prevotella melaninogenica*).

Early interest in *Porphyromonas gingivalis* and other black-pigmented *Bacteroides* arose primarily because of their essential role in certain experimental mixed infections (Macdonald *et al*. 1956, 1963; Socransky & Gibbons 1965) and their production of an unusually large array of virulence factors (Table 9-2) (Haffajee & Socransky 1994; Deshpande & Khan 1999; Holt & Ebersole 2005). Members of these species produce collagenase, gingipain, an array of proteases (including those that destroy immunoglobulins), hemolysins, endotoxin, fatty acids, ammonia, hydrogen sulfide, indole etc. *P. gingivalis* can inhibit migration of PMNs across an epithelial barrier (Madianos *et al*. 1997), has been shown to affect the production or degradation of cytokines by mammalian cells (Darveau *et al*. 1998; Fletcher *et al*. 1998; Sandros *et al*. 2000). and produces extracellular vesicles that contribute to the loss of membrane-bound CD14 receptors on human macrophage-like cells (Duncan *et al*. 2004).

Studies initiated in the late 1970s and extending to the present have strengthened the association of *P. gingivalis* with disease and demonstrated that the species is uncommon and in low numbers in health or gingivitis but more frequently detected in destructive forms of disease (Table 9-2) (Haffajee & Socransky 1994; O'Brien-Simpson *et al*. 2000; Takeuchi *et al*. 2001; van Winkelhoff *et al*. 2002; Lau *et al*. 2004; Yang *et al*. 2004b). In diseased subjects, there was a strong positive relationship with pocket depth (Kawada *et al*. 2004; Socransky & Haffajee 2005). This species has also been shown to be increased in numbers and or frequency of detection in deteriorating periodontal sites (Dzink *et al*. 1988; Lopez 2000; Kamma *et al*. 2001) or in subjects exhibiting periodontal disease progression (Albandar *et al*. 1997). The species has been shown to be reduced in successfully treated sites but was commonly encountered in sites that exhibited recurrence of disease or persistence of deep periodontal pockets post-therapy (Bragd *et al*. 1987; Haffajee *et al*. 1988a; van Winkelhoff *et al*. 1988; Berglundh *et al*. 1998; Shiloah *et al*. 1998; Winkel *et al*. 1998; Takamatsu *et al*. 1999; Chaves *et al*. 2000; Mombelli *et al*. 2000; Fujise *et al*. 2002; Kawada *et al*. 2004). *P. gingivalis* has been associated with an increased risk of periodontal disease severity and progression (Beck *et al*. 1990, 1992, 1997; Grossi *et al*. 1994, 1995).

P. gingivalis has been shown to induce elevated systemic and local immune responses in subjects with various forms of periodontitis (Table 9-2) (Haffajee & Socransky 1994; Mahanonda *et al*. 1991; O'Brien-Simpson *et al*. 2000). Indeed, there has been an effort in many laboratories, not only to compare the level of antibody response in subjects with and without disease, but to examine relative avidities of antibody (Lopatin & Blackburn 1992; Whitney *et al*. 1992; Mooney *et al*. 1993), subclass of antibody (Lopatin & Blackburn 1992; Wilton *et al*. 1992), the effect of treatment (Chen *et al*. 1991; Johnson *et al*. 1993), and the nature of the antigens which elicit the elevated responses (Ogawa *et al*. 1989; Yoshimura *et al*. 1989; Curtis *et al*. 1991; Papaioannou *et al*. 1991; Duncan *et al*. 1992; Schifferle *et al*. 1993). Noteworthy in this regard were the observations of Ogawa *et al*. (1989), which indicated that an average of approximately 5% of plasma cells in lesions of advanced periodontitis formed antibody to the fimbriae of *P. gingivalis*. The consensus of the antibody studies is that many, but not all, subjects who had experienced periodontal attachment loss exhibited elevated levels of antibody to antigens of *P. gingivalis*, suggesting that this species gained access to the underlying tissues and may have initiated or contributed to the observed pathology.

P. gingivalis-like organisms were also strongly related to destructive periodontal disease in naturally occurring or ligature-induced disease in dogs, sheep or monkeys (Table 9-2). The species or closely related organisms were higher in number in lesion sites than in non-lesion sites in naturally occurring disease. When disease was induced by ligature in dogs or monkeys, the level of the species rose at the diseased sites concomitant with the detection of disease. Of great interest were the observations of Holt *et al*. (1988) who demonstrated that a microbiota suppressed by systemic administration of rifampin (and without detectable *P. gingivalis*) would not cause ligature-induced disease, but the re-introduction of *P. gingivalis* to the microbiota resulted in initiation and progress of the lesions. Ligature-induced periodontitis and peri-implantitis in dogs was also accompanied by a significant increase in the detection of *P. gingivalis* (Nociti *et al*. 2001). Like *A. actinomycetemcomitans*, *P. gingivalis* has been shown to be able to invade human gingival epithelial cells *in vitro* (Lamont *et al*. 1992; Duncan *et al*. 1993; Sandros *et al*. 1993), buccal epithelial cells *in vivo* (Rudney *et al*. 2001), endothelial cells (Takahashi *et al*. 2006) and has been found in higher numbers on or in epithelial cells recovered from the periodontal pocket than in associated plaque (Dzink *et al*. 1989) or healthy sites (Colombo *et al*. 2006). Attachment to and invasion of epithelial cells appears to be mediated by the *P. gingivalis* fimbriae (Njoroge *et al*. 1997; Weinberg *et al*. 1997; Nakajawa *et al*. 2006).

There have been several studies that have attempted to immunize experimental animals against periodontal disease induced by *P. gingivalis*. Studies in monkeys and gnotobiotic rats have indicated that immunization with whole organisms or specific antigens affected the progress of the periodontal lesions. In most instances, periodontal breakdown was

decreased (Evans *et al*. 1992; Persson *et al*. 1994a). However, in one study, the disease severity was increased after immunization (Ebersole *et al*. 1991). In the monkey model, the percentage of *P. gingivalis* cells in subgingival plaque was inversely related to the serum antibody titer to this species (Persson *et al*. 1994b). Reductions in alveolar bone loss in the monkey model could also be achieved by immunization with the cysteine protease porphypain-2 from *P. gingivalis* (Moritz *et al*. 1998; Page 2000). In more recent years, investigators have used a mouse "oral challenge" (by cells of *P. gingivalis*) model to study the effects of immunization by various fractions of *P. gingivalis* on alveolar bone loss induced by this species. Immunization by hemagglutinin B (Katz *et al*. 1999), capsular polysaccharide (Gonzalez *et al*. 2003), heat shock protein (Lee *et al*. 2006), gingipain R (Gibson & Genco 2001), and the active sites of RgpA and Kgp proteinases (O'Brien-Simpson *et al*. 2005) protected against alveolar bone loss in the mouse model. Thus, altering the host–*P. gingivalis* equilibrium by raising the level of specific antibodies to *P. gingivalis* antigens markedly affected disease outcome. Such data reinforce the importance of this bacterial species in periodontal disease, at least in the animal model systems employed.

Tannerella forsythia

The third consensus periodontal pathogen, *T. forsythia*, was first described in 1979 (Tanner *et al*. 1979) as a "fusiform" *Bacteroides*. This species was difficult to grow, often requiring 7–14 days for minute colonies to develop. The organism is a Gram-negative, anaerobic, spindle-shaped, highly pleomorphic rod. The growth of the organism was shown to be enhanced by co-cultivation with *F. nucleatum* and indeed it commonly occurred with this species in subgingival sites (Socransky *et al*. 1988). The need for co-cultivation could be overcome by providing N-acetylmuramic acid in the medium (Wyss 1989). Inclusion of this factor markedly enhanced growth and the resulting cells were regularly shaped, short, Gram-negative rods rather than the pleomorphic cells observed in the absence of this factor (Tanner & Izard 2006). A feature that *T. forsythia* cells shares with certain other Gram-negative species is the presence of a serrated S-layer that is easily visible by electron microscopy (Tanner *et al*. 1986) that may contribute to the pathogenicity of the species in periodontal diseases (Sabet *et al*. 2003). The S-layer has been isolated and shown to mediate hemagglutination, adhesion/invasion of epithelial cells, and murine subcutaneous abscess formation. The S-layer is composed of two glycoproteins of molecular mass 200 and 210 kDa (Lee *et al*. 2006). This species has been shown to produce trypsin-like proteolytic activity (BANA test positive) (Loesche *et al*. 1992) and methylglyoxal (Kashket *et al*. 2002), and induce apoptotic cell death (Arakawa *et al*. 2000). In addition, *T. forsythia* in co-cultures of macrophage and epithelial cells leads to the expression of pro-inflammatory cytokines, chemokines, PGE_2, and MMP9 (Bodet *et al*. 2006).

Initially, *T. forsythia* was thought to be a relatively uncommon subgingival species. However, the studies of Gmur *et al*. (1989) using monoclonal antibodies to enumerate the species directly in plaque samples, suggested that the species was more common than previously found in cultural studies and its levels were strongly related to increasing pocket depth. Lai *et al*. (1987) reported similar findings using fluorescent-labeled polyclonal antisera and demonstrated that *T. forsythia* was much higher in subgingival than supragingival plaque samples. Data of Tanner *et al*. (1998) suggested that *T. forsythia* was a major species found at sites that converted from periodontal health to disease. There was a greater risk of periodontal attachment loss in adolescents who were colonized by *T. forsythia* than adolescents in whom the species was not detected (Hamlet *et al*. 2004). *T. forsythia* was in much higher counts, proportions, and prevalence in subjects with various forms of periodontitis than in periodontally healthy subjects (van Winkelhoff *et al*. 2002; Yang *et al*. 2004b; Haffajee *et al*. 2006a). *T. forsythia* was found in higher numbers in sites of destructive periodontal disease or periodontal abscesses than in gingivitis or healthy sites (Lai *et al*. 1987; Herrera *et al*. 2000; Papapanou *et al*. 2000; Lau *et al*. 2004). In addition, *T. forsythia* was detected more frequently and in higher numbers in actively progressing periodontal lesions than inactive lesions (Dzink *et al*. 1988) (Table 9-3). Further, subjects who harbored *T. forsythia* were at greater risk for alveolar bone loss, attachment loss and tooth loss compared with subjects in whom this species was not detected (Machtei *et al*. 1999).

Since these early studies, a large number of additional studies have demonstrated the association of *T. forsythia* with periodontal disease using techniques such as PCR and DNA hybridization (Tanner & Izard 2006). *T. forsythia* has also been shown to be present in the oral cavities of monkeys, cats, and dogs, and species related to *T. forsythia* have been found in insects such as termites (Tanner & Izard 2006). An as yet uncultivated clone similar to *T. forsythia* has been found more frequently in subjects who were periodontally healthy than subjects with periodontitis (Leys *et al*. 2002).

T. forsythia has been shown to be decreased in frequency of detection and counts after successful periodontal therapy, including scaling and root planing (SRP) (Haffajee *et al*. 1997; Takamatsu *et al*. 1999; Cugini *et al*. 2000; Darby *et al*. 2001, 2005; van der Velden *et al*. 2003; Teles *et al*. 2006), periodontal surgery (Levy *et al*. 2002), or systemically administered antibiotics (Feres *et al*. 2000; Winkel *et al*. 1998, 2001; Haffajee *et al*. 2006b; Teles *et al*. 2006). *T. forsythia* was found at higher levels at sites which showed breakdown after periodontal therapy than

sites which remained stable or gained attachment (Shiloah *et al*. 1998; Fujise *et al*. 2002). Ligature-induced periodontitis and peri-implantitis in dogs were accompanied by a significant increase in the frequency of detection of *T. forsythia* (Nociti *et al*. 2001). Finally, subjects with a low severity of chronic periodontitis who exhibited a persistent presence of *T. forsythia* at periodontal sites had a 5.3 times greater chance of having at least one site in their mouths losing attachment compared with subjects with occasional or no presence of this species (Tran *et al*. 2001).

Studies using checkerboard DNA–DNA hybridization techniques to examine subgingival plaque samples not only confirmed the high levels of *T. forsythia* detected using fluorescent-labeled antisera but demonstrated that *T. forsythia* was the most common species detected on or in epithelial cells recovered from periodontal pockets (Dibart *et al*. 1998). It was infrequently detected in epithelial cell samples from healthy subjects. Double-labeling experiments demonstrated that *T. forsythia* was both on and in periodontal pocket epithelial cells and indicated the species' ability to invade. Listgarten *et al*. (1993) found that the species most frequently detected in "refractory" subjects was *T. forsythia.* Serum antibody to *T. forsythia* has been found to be elevated in a number of periodontitis patients (Taubman *et al*. 1992) and was often extremely elevated in a subset of refractory periodontal disease subjects. The observation that *T. forsythia* shares antigens with *P. gingivalis* suggests that protective antibody formed to one species may provide protection against both species (Vasel *et al*. 1996).

The role of *T. forsythia* in periodontal diseases has been clarified and strengthened by studies in numerous laboratories involving non-cultural methods of enumeration, such as DNA probes, PCR or immunologic methods. For example, Grossi *et al*. (1994, 1995) considered *T. forsythia* to be the most significant microbial risk factor that distinguished subjects with periodontitis from those who were periodontally healthy.

Spirochetes

Spirochetes are Gram-negative, anaerobic, helical-shaped, highly motile microorganisms that are common in many periodontal pockets (Fig. 9-4). The role of spirochetes in the pathogenesis of destructive periodontal diseases deserves extended comment. Clearly, a spirochete has been implicated as the likely etiologic agent of acute necrotizing ulcerative gingivitis by its presence in large numbers in tissue biopsy specimens from affected sites (Listgarten & Socransky 1964; Listgarten 1965). The role of spirochetes in other forms of periodontal disease is less clear. The organisms have been considered as possible periodontal pathogens since the late 1800s and in the 1980s enjoyed a resurgence of interest for use as possible diagnostic indicators of disease activity and/or therapeutic efficacy (Keyes & Rams 1983; Rams & Keyes 1983). The major reason for the interest in this group of organisms has been their increased numbers in sites with increased pocket depth. Healthy sites exhibit few, if any, spirochetes, sites of gingivitis but no attachment loss exhibit low to moderate levels, while many deep pockets harbor large numbers of these organisms. Further, spirochetes such as *Treponema denticola*, have been shown to be at the forefront of periodontal lesions as demonstrated in sections of undisturbed subgingival plaque using immunohistochemical localization (Kigure *et al*. 1995; Noiri *et al*. 2001). The localization of spirochetes next to the epithelial lining of the periodontal pocket may facilitate both attachment of these species to epithelial cells and invasion into the adjacent tissues.

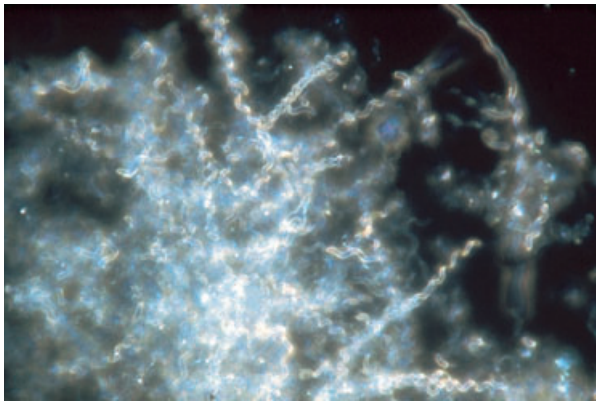

Fig. 9-4 Photomicrograph of a sample of subgingival plaque from a subject with advanced chronic periodontitis viewed by darkfield microscopy. The sample was dominated by large spirochetes with the typical corkscrew appearance.

The major difficulty encountered in defining the role of spirochetes has been the difficulty in distinguishing individual species. This is due in large part to difficulty in cultivating spirochetes in general and, in particular, species of spirochetes that are currently uncultivable. There are currently 10 cultivable species of spirochetes (Ellen & Galimanas 2005). At least 50 taxa of subgingival spirochetes can be recognized using 16S rRNA analysis (Dewhirst *et al*. 2000). The cultivable spirochetes require very complex media for their cultivation. These contain infusions of animal organs, trypsin digests of casein, various fatty acids and accessory growth factors, and serum (Ellen & Galimanas 2005). Wyss *et al*. (1999) have developed more defined media for the cultivation of some of the species of oral spirochetes. In spite of the ability to cultivate certain species of oral spirochetes, it has been difficult to use these media to enumerate the spirochetes in subgingival plaque samples. Therefore, in many of the earlier studies of plaque samples, spirochetes were combined either in a single group or groups based on cell size; i.e. small, medium or large. Thus, while there may be pathogens among the spirochetes, their role may have been obscured by unintentionally pooling their numbers with

non-pathogenic spirochetes. This would be similar to combining in a single count, organisms with coccal morphologies, such as *P. gingivalis*, *Veillonella parvula*, and *Streptococcus sanguinis*.

In spite of the limitations of combining spirochetes into a single morphogroup, spirochetes as a group or as individual species have been related to periodontal disease (Ellen & Galimanas 2005). Spirochetes have been associated with an increased risk at a site for the development of gingivitis (Riviere & DeRouen 1998) and periodontitis (Riviere *et al*. 1997). The need to evaluate the role of individual species of spirochetes in periodontal diseases is reinforced by studies of serum antibody responses to different species. When antibody responses to individual species were examined in subjects with chronic or aggressive periodontitis or a healthy periodontium, different responses were observed to different species. Certain spirochetal species elicited an elevated response in one or more of the groups with destructive periodontal disease (Mangan *et al*. 1982; Tew *et al*. 1985c; Lai *et al*. 1986), while others were related to depressed antibody responses in certain patient groups (Steinberg & Gershoff 1968; Tew *et al*. 1985c). Such data suggest that pooling spirochete species into a collective group may obscure meaningful host–parasite interactions.

More recently, specific species of spirochetes have been related to periodontal breakdown using antibody-based or molecular techniques. *Treponema denticola* was found to be more common in periodontally diseased than healthy sites, more common in subgingival than supragingival plaque (Simonson *et al*. 1988; Riviere *et al*. 1992; Albandar *et al*. 1997; Haffajee *et al*. 1998; Yuan *et al*. 2001), and more common in healthy sites that progressed to gingivitis (Riviere & DeRouen 1998). *Tr. denticola* was shown to decrease in successfully treated periodontal sites, but not change or increase in non-responding sites (Simonson *et al*. 1992). Cultural studies suggested that *Tr. denticola* and a "large treponeme" were found more frequently in patients with severe periodontitis than in healthy or gingivitis sites (Moore *et al*. 1982).

Riviere *et al*. (1991,a,b,c, 1992) employed a monoclonal antibody directed against *Treponema pallidum*, the etiologic agent of syphilis, to examine supra- and subgingival plaque samples and/or tissues from healthy, periodontitis and ANUG subjects. This antibody cross-reacted with antigens of uncultivated spirochetes in many of the plaque samples. These "pathogen-related oral spirochetes" (PROS) were the most frequently detected spirochetes in supra- and subgingival plaques of periodontitis patients and were the most numerous spirochetes in periodontitis lesion sites. Their presence in periodontally healthy sites was related to an increased risk of development of periodontitis (Riviere *et al*. 1997). The PROS were also detected in plaque samples from ANUG (Riviere *et al*. 1991c) and tissue biopsies from ANUG lesions using immunohistochemical techniques (Riviere *et al*. 1991a). PROS were also shown to have the ability to penetrate a tissue barrier in *in vitro* systems (Riviere *et al*. 1991b). This property was shared with *Tr. pallidum* but not with other cultivated species of oral spirochetes such as *Tr. denticola*, *Tr. socranskii*, *Tr. pectinovorum* or *Tr. vincentii*. In later studies, the PROS were shown by molecular techniques to share 16S rRNA gene sequences with *Tr. vincentii* and *Tr. medium* (Choi *et al*. 1996; Riviere *et al*. 1999). These studies and others suggested that certain specific species of spirochetes were important in the pathogenesis of ANUG and certain forms of periodontitis. Precise evaluation of the role of individual spirochete species appears to be realistic based on their detection in plaque samples by immunologic, PCR or DNA probe techniques. Indeed, enumeration of even uncultivable spirochete taxa is possible using oligonucleotide probes (Tanner *et al*. 1994) or specific antibody as described above. Studies performed using such techniques permit better distinction of species of spirochetes and a clearer understanding of their possible role in disease.

The mechanisms of pathogenicity of the spirochetes have been under active investigation in recent years. Spirochetes demonstrate pathogenicity in animal abscess model systems (Kesavalu *et al*. 1999; Kimizuka *et al*. 2003), and produce a wide range of potential virulence factors (Ellen 2005). Among the virulence factors that may play a major role is a subtilisin family protease, dentilisin, that is encoded by the *prtP* gene. This enzyme affects a wide range of protein substrates including fibronectin, laminin, and fibrinogen (Ishihara *et al*. 1996). It is thought that spirochetes may prolong tissue remodeling and wound healing following periodontal treatment; thus, the chronic periodontal lesion may represent an "ever-healing" wound that is sustained during chronic infection (Ellen & Galimanas, 2005).

Successful treatment of periodontal infections is accompanied by a decrease in the numbers and proportions of oral spirochetes as a group and individual species. Indeed, this reduction is so consistent that it has been used in some studies as a measure of compliance in determining whether subjects used the prescribed antibiotics (Loesche *et al*. 1993).

Prevotella intermedia/Prevotella nigrescens

At present the data for other species as etiologic agents of destructive periodontal diseases are more limited, but certain organisms appear to merit further investigation (Zambon 1996). *Pr. intermedia* is the second black-pigmented *Bacteroides* to receive considerable interest (Fig. 9-5). The levels of this Gram-negative, short, round-ended anaerobic rod have been shown to be particularly elevated in acute necrotizing ulcerative gingivitis (Loesche *et al*. 1982), certain forms of periodontitis (Tanner *et al*. 1979; Dzink *et al*. 1983; Moore *et al*. 1985; Maeda *et al*. 1998; Herrera *et al*. 2000; Papapanou *et al*. 2000; Lee *et al*.

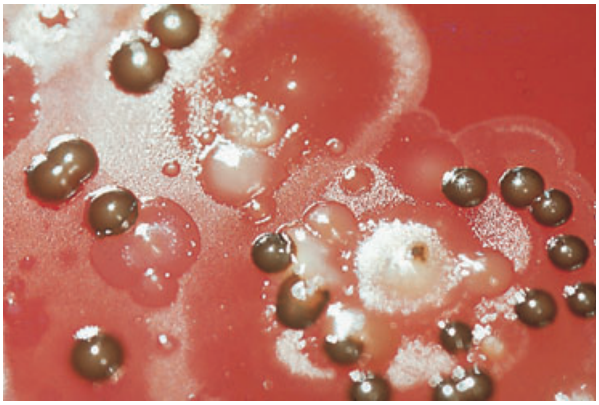

Fig. 9-5 Photograph of part of a primary isolation plate of a subgingival plaque sample from a subject with chronic periodontitis. The medium and growth conditions were as described in Fig. 9-2. The dark-pigmented colonies were isolates of *Prevotella intermedia*.

2003; van Winkelhoff *et al*. 2002; Alves *et al* 2006; Boutaga *et al*. 2006), and progressing sites in chronic periodontitis (Tanner *et al*. 1996; Lopez 2000), and it has been detected by immunohistological methods in the intercellular spaces of periodontal pocket biopsies from rapidly progressive periodontitis subjects (Hillmann *et al*. 1998). Isolates of this species can induce alveolar bone loss in rats (Yoshida-Minami *et al*. 1997). *Pr. intermedia* was reduced more markedly in subgingival plaque samples from subjects who received adjunctive systemically administered amoxicillin plus metronidazole than subjects receiving a placebo (Rooney *et al*. 2002). Persistence of *Pr. intermedia/nigrescens* after standard mechanical therapy has been shown to be associated with a large proportion of sites exhibiting bleeding on probing (Mombelli *et al*. 2000). Berglundh *et al*. (1998) demonstrated that improved clinical parameters after the use of mechanical therapy and systemically administered amoxicillin and metronidazole were associated with a decrease of periodontal pathogens including *Pr. intermedia.*

This species appears to have a number of the virulence properties exhibited by *P. gingivalis* and was shown to induce mixed infections on injection in laboratory animals (Hafstrom & Dahlen 1997). Like *P. gingivalis*, *Pr. intermedia/nigrescens* appears to induce an increased release of MMP-8 and MMP-9 in gingival pockets as well as MMP-9 in plasma (Soder *et al*. 2006). It has also been shown to invade oral epithelial cells *in vitro* (Dorn *et al*. 1998) and induce expression of nitric oxide synthase in tissue culture cells (Kim *et al*. 2004). Elevated seroantibodies to this species have been observed in some but not all subjects with refractory periodontitis (Haffajee *et al*. 1988b). Elevated IgG antibody to *Pr. intermedia* was associated with coronary heart disease (CHD) in past and current smokers, while elevated IgG antibody to *Pr. nigrescens* was associated with CHD in never smokers (Beck *et al*. 2005). Strains of *Pr. intermedia* that show identical phenotypic traits have been separated into two species, *Pr. intermedia* and *Pr. nigrescens* (Shah & Gharbia 1992). This distinction makes earlier studies of this "species" difficult to interpret since data from two different species may have been inadvertently pooled. However, new studies which discriminate the species in subgingival plaque samples might strengthen the relationship of one or both species to periodontal disease pathogenesis.

Fusobacterium nucleatum

F. nucleatum is a Gram-negative, anaerobic, spindle-shaped rod that has been recognized as part of the subgingival microbiota for over 100 years (Plaut 1894; Vincent 1899). This species was the most common isolate found in cultural studies of subgingival plaque samples, comprising approximately 7–10% of total isolates from different clinical conditions (Dzink *et al*. 1985, 1988; Moore *et al*. 1985). *F. nucleatum* was prevalent in subjects with periodontitis (Papapanou *et al*. 2000; Colombo *et al*. 2002; Socransky *et al*. 2002; Boutaga *et al*. 2006) and periodontal abscesses (Herrera *et al*. 2000) and was reduced after successful periodontal therapy (van der Velden *et al*. 2003; Haffajee *et al*. 2006b). Although there were differences detected in levels of this species between active and inactive periodontal lesions (Dzink *et al*. 1988), the differences may have been minimized by the inadvertent pooling of subspecies of *F. nucleatum*. Support for this contention may be derived from the antibody responses in subjects with different forms of periodontal disease to different homology groups of *F. nucleatum* (Tew *et al*. 1985b). The role of *F. nucleatum* in periodontal diseases is being clarified by examining the relationship of individual subspecies, such as *F. nucleatum* ss *nucleatum*, *F. nucleatum* ss *polymorphum*, *F. nucleatum* ss *vincentii*, and *F. periodonticum*, to disease status and progression.

IgG and IgM titers in serum against the lipopolysaccharide (LPS) of *F. nucleatum* were higher in subjects with periodontitis than in healthy individuals (Onoue *et al*. 2003). Invasion of this species into human gingival epithelial cells *in vitro* was accompanied by an increased secretion of IL-8 from the epithelial cells (Han *et al*. 2000). The species can induce apoptotic cell death in mononuclear and polymorphonuclear cells (Jewett *et al*. 2001), induces epithelial cells to produce collagenase 3 (Uitto *et al*. 2005), and produces a 65 kDa serine protease (Bachrach *et al*. 2004). In addition, *F. nucleatum* induces cytokine, elastase, and oxygen radical release from leukocytes (Sheikhi *et al*. 2000). Perhaps the most important role of *F. nucleatum* in the subgingival ecosystem is its function as a "bridging" species, facilitating coaggregation among species as described below.

Campylobacter rectus

C. rectus is a Gram-negative, anaerobic, short, motile vibrio. The organism is unusual in that it utilizes

hydrogen or formate as its energy source. It was first described as a member of the "vibrio corroders", a group of short nondescript rods that formed small convex, "dry spreading" or "corroding" (pitting) colonies on blood agar plates. These organisms were eventually shown to include members of a new genus *Wolinella* (most species have been redefined as *Campylobacter*), and *Eikenella corrodens*. *C. rectus* has a 150-kDa protein on its cell surface that forms a paracrystalline lattice or S-layer that surrounds the bacterium (Wang *et al*. 1998, 2000). *C. rectus* may help to initiate periodontitis by increasing the expression of proinflammatory cytokines and the S-layer may help to moderate this response facilitating the survival of the species at the site of infection. *C. rectus* is widely distributed in subgingival sites, even in the primary, mixed and permanent dentitions of children (Umeda *et al*. 2004; Hayashi *et al*. 2006). *C. rectus* has been shown to be present in higher numbers in samples from diseased sites as compared with healthy sites (Moore *et al*. 1983, 1985; Lippke *et al*. 1991; Lai *et al*. 1992; Papapanou *et al*. 1997; Macuch & Tanner 2000; Dogan *et al*. 2003; Ihara *et al*. 2003; Suda *et al*. 2004; Nonnenmacher *et al*. 2005) and it was found in higher numbers and more frequently at sites exhibiting active periodontal destruction (Dzink *et al*. 1985, 1988; Tanner & Bouldin 1989; Rams *et al*. 1993) or converting from periodontal health to disease (Tanner *et al*. 1998). In addition, *C. rectus* was found less frequently and in lower numbers after successful periodontal therapy (Tanner *et al*. 1987; Haffajee *et al*. 1988a; Levy *et al*. 1999; Colombo *et al*. 2005). *C. rectus* was also found in combination with other suspected pathogens in sites of subjects with refractory periodontal diseases (Haffajee *et al*. 1988b) and was in higher levels in subjects with aggressive periodontitis than in subjects with other forms of periodontitis (Gajardo *et al*. 2005). Like *A. actinomycetemcomitans*, *C. rectus* has been shown to produce a leukotoxin. These are the only two oral species known to possess this characteristic (Gillespie *et al*. 1992). The species is also capable of stimulating human gingival fibroblasts to produce IL-6 and IL-8 (Dongari-Bagtzoglou & Ebersole 1996). Higher serum antibody levels to *C. rectus* GroEL was detected in patients with periodontitis when compared with control subjects (Fukui *et al*. 2006).

C. rectus has been associated with a number of systemic conditions. Elevated IgM antibody to *C. rectus* in fetal chord blood has been associated with an increased rate of prematurity (Madianos *et al*. 2001) and increased levels of *C. rectus* along with *Peptostreptococcus micros* in subgingival plaque samples of pregnant females was associated with an increased risk of pre-term low birth weight (Buduneli *et al*. 2005). IgG antibody to these same two species was also associated with increased carotid intima–medial thickness (Beck *et al*. 2005). Finally, *C. rectus*, as well as other oral species, has been detected in atherosclerotic vessels (Fiehn *et al*. 2005) and in occluded arteries in patients with Buerger disease (Iwai *et al*. 2005).

Eikenella corrodens

E. corrodens is a Gram-negative, capnophilic, asaccharolytic, regular, small rod with blunt ends. It has been recognized as a pathogen in other forms of disease, particularly osteomyelitis (Johnson & Pankey 1976), infections of the central nervous system (Emmerson & Mills 1978; Brill *et al*. 1982), and root canal infections (Goodman 1977). This species was found more frequently in sites of periodontal destruction as compared with healthy sites in some (Savitt & Socransky 1984; Muller *et al*. 1997; Yuan *et al*. 2001), but not all studies (Papapanou *et al*. 2000). In addition, *E. corrodens* was found more frequently and in higher levels in actively breaking down periodontal sites (Dzink *et al*. 1985; Tanner *et al*. 1987) and in sites of subjects who responded poorly to periodontal therapy (Haffajee *et al*. 1988b). Successfully treated sites harbored lower proportions of this species (Tanner *et al*. 1987). *E. corrodens* has been found to be elevated in lesions in LAP subjects (Suda *et al*. 2002) as well as in association with *A. actinomycetemcomitans* in such lesions (Mandell 1984; Mandell *et al*. 1987). In tissue culture systems, *E. corrodens* has been shown to stimulate the production of matrix metalloproteinases (Dahan *et al*. 2001) and IL-6 and IL-8 (Yumoto *et al*. 1999, 2001). While there is some association of this species with periodontal disease, to date it has not been particularly strong (Chen *et al*. 1989).

Peptostreptococcus micros

Pe. micros is a Gram-positive, anaerobic, small, asaccharolytic coccus. It has long been associated with mixed anaerobic infections in the oral cavity and other parts of the body (Finegold 1977). Two genotypes can be distinguished, with the smooth genotype being more frequently associated with periodontitis lesions than the rough genotype (Kremer *et al*. 2000). *Pe. micros* has been detected more frequently and in higher numbers at sites of periodontal destruction as compared with gingivitis or healthy sites (Moore *et al*. 1983, 1985; Herrera *et al*. 2000; Papapanou *et al*. 2000; Choi *et al*. 2000; Riggio *et al*. 2001; van Winkelhoff *et al*. 2002; Lee *et al*. 2003; Nonnenmacher *et al*. 2005; Boutaga *et al*. 2006; Gomes *et al*. 2006), was elevated in actively breaking down sites (Dzink *et al*. 1988), and at higher mean levels in current smokers compared with non-smokers (van Winkelhoff *et al*. 2001). The levels and frequency of detection of the species were decreased at successfully treated periodontal sites (Haffajee *et al*. 1988a). Studies of systemic antibody responses to suspected periodontal pathogens indicated that subjects with severe generalized periodontitis had elevated antibody levels to this species when compared with

healthy subjects or subjects with LAP (Tew *et al*. 1985a). *Pe. micros* produces proteases (Grenier & Bouclin 2006) and, in a mouse skin model system, it was shown that this species in combination with either *Pr. intermedia* or *Pr. nigrescens* could produce transmissible abscesses (van Dalen *et al*. 1998). In a study of chronic periodontitis subjects with and without acute myocardial infarction, it was found that *Pe. micros* was much higher in the plaque samples of the subjects exhibiting myocardial infarction (Dogan *et al*. 2005).

Selenomonas species

Selenomonas species have been observed in plaque samples using light microscopy for many decades. The organisms may be recognized by their curved shape, tumbling motility, and, in good preparations, by the presence of a tuft of flagella inserted in the concave side. The *Selenomonas* spp. are Gram-negative, curved, saccharolytic rods. The organisms have been somewhat difficult to grow and speciate. However, Moore *et al*. (1987) described six genetically and phenotypically distinct groups isolated from the human oral cavity. *Selenomonas noxia* was found at a higher proportion of shallow sites (pocket depth (PD) <4 mm) in chronic periodontitis subjects compared with similar sites in periodontally healthy subjects (Haffajee *et al*. 1998). Further, *S. noxia* was found to be associated with sites that converted from periodontal health to disease (Tanner *et al*. 1998).

Eubacterium species

Certain *Eubacterium* species have been suggested as possible periodontal pathogens due to their increased levels in diseased sites, particularly those of severe periodontitis (Uematsu & Hoshino 1992). *E. nodatum*, *Eubacterium brachy*, and *Eubacterium timidum* are Gram-positive strictly anaerobic, small somewhat pleomorphic rods. They are often difficult to cultivate, particularly on primary isolation, and appear to grow better in roll tubes than on blood agar plates. To date, there is greater evidence supporting a possible etiologic role in periodontitis for *E. nodatum* than the other *Eubacterium* species. Moore *et al*. (1982, 1985) used the roll tube cultural technique to examine the proportions of bacterial species in subgingival plaque samples from subjects with various forms of periodontitis, gingivitis, and health. They found that *E. nodatum* was absent or in low proportions in periodontal health and various forms of gingivitis, but was present in higher proportions in moderate periodontitis (2%), generalized early onset periodontitis (8%), LAP (6%), early onset periodontitis (5%), and adult (chronic) periodontitis (2%). *E. nodatum* was in the top 2–14 species enumerated in these different periodontal states. Uematsu and Hoshina (1992) found *Eubacterium* species to be the predominant species in subgingival plaque samples from subjects with advanced periodontitis using cultural techniques. More recent studies have confirmed an association of *E. nodatum* with periodontitis using molecular techniques. Using species-specific oligonucleotide probes, Booth *et al*. (2004) found that *E. nodatum* was at significantly higher counts in patients than in matched control subjects. The species was also at higher levels in deep compared with shallow pockets. Papapanou *et al*. (2000) found higher counts of *E. nodatum* in 131 periodontitis patients than in 74 periodontally intact controls using checkerboard DNA–DNA hybridization. Colombo *et al*. (2002) also used checkerboard DNA–DNA hybridization to evaluate the microbiota in 25 untreated Brazilian subjects with chronic periodontitis and found a significant positive correlation of *E. nodatum* with mean pocket depth and attachment level. Samples of subgingival plaque were taken from 21 832 periodontal sites in 635 chronic periodontitis and 189 periodontally healthy subjects and examined by checkerboard DNA–DNA hybridization (Haffajee *et al*. 2006a). It was found that *E. nodatum* was strongly associated with chronic periodontitis both in the presence of high levels of *P. gingivalis* and *T. forsythia* and in subjects where these species were in lower proportions. It has also been demonstrated that the percentage of sites colonized by *E. nodatum* was significantly higher in current smokers than non-smokers (Haffajee & Socransky 2001). Some of the *Eubacterium* species elicited elevated antibody responses in subjects with different forms of destructive periodontitis (Tew *et al*. 1985a,b; Vincent *et al*. 1986; Martin *et al*. 1988).

The "milleri" streptococci

Streptococci were frequently implicated as possible etiologic agents of destructive periodontal diseases in the early part of the twentieth century. Cultural studies of the last 2 decades have also suggested the possibility that some of the streptococcal species were associated with, and may contribute to, disease progression. At this time, evidence suggests that the "milleri" streptococci, *Streptococcus anginosus*, *Streptococcus constellatus*, and *S. intermedius*, might contribute to disease progression in subsets of periodontal patients. The species was found to be elevated at sites which demonstrated recent disease progression (Dzink *et al*. 1988). Walker *et al*. (1993) found *S. intermedius* to be elevated in a subset of patients with refractory disease at periodontal sites which exhibited disease progression. Colombo *et al* (1998a) found that subjects exhibiting a poor response to SRP and then to periodontal surgery with systemically administered tetracycline had higher levels and proportions of *S. constellatus*, than subjects who responded well to periodontal therapy. Refractory subjects also exhibited elevated serum antibody to *S. constellatus* when compared with successfully treated subjects (Colombo *et al*. 1998b). In a study of 161 subjects with

acute coronary syndrome (ACS) and 161 control subjects, it was suggested that the oral bacterial load of species including *S. intermedius* and *S. anginosus* may be a risk factor for ACS (Renvert *et al*. 2006). The data on streptococci are somewhat limited, but a continued examination of their role in disease seems warranted.

Other species

It has long been recognized that many taxa in subgingival plaque were not being cultivated based on microscopic observations that revealed cell morphotypes that were never recovered in culture. In addition, there were marked differences between total viable counts (representing cultivable species) and total microscopic counts representing all organisms (Socransky *et al*. 1963; Olsen & Socransky 1981; Moore & Moore 1994). Currently, the best model for exploring microbial diversity is based on isolating DNA from the target environment, amplifying the rDNA using consensus primers and PCR, cloning the amplicons into *Escherichia coli*, and sequencing the cloned 16S rDNA inserts (Pace *et al*. 1986; Hugenholtz & Pace 1996). The resulting sequences are compared with those of known species and phylotypes in sequence databases, such as GenBank and the Ribosomal Database Project (Cole *et al*. 2005). These culture-independent molecular phylogenetic methods have been used to deduce the identity of novel phylotypes from periodontitis subjects (Choi *et al*. 1994; Spratt *et al*. 1999). To date, based on sequence analysis of 16S rRNA clonal libraries from specimens of the oral cavity, over 700 bacterial taxa have been detected, of which over half have not yet been cultivated *in vitro* (Dewhirst *et al*. 2000; Paster *et al*. 2001, 2002; Becker *et al*. 2002; Kazor *et al*. 2003). "New" putative pathogens were tentatively identified in a study in which the presence of 39 bacterial species were determined that were implicated in health or disease based on 16S rRNA clonal analysis. Samples from 66 subjects with chronic periodontitis and 66 age-matched healthy controls were examined for the presence of target species. Associations and relative risks were determined for these species. Several novel taxa, in addition to the classical putative pathogens, were suggested as potential periodontal pathogens or health-related species (Kumar *et al*. 2003).

Interest has grown in groups of cultivable species not commonly found in the subgingival plaque as initiators or possibly contributors to the pathogenesis of periodontal disease, particularly in individuals who have responded poorly to periodontal therapy. Species not commonly thought to be present in subgingival plaque can be found in a proportion of such subjects or even in subjects who have not received periodontal treatment. Studies have examined enteric organisms and staphylococcal species as well as other unusual mouth inhabitants. Slots *et al*. (1990b) used cultural techniques to examine plaque samples from over 3000 chronic periodontitis patients and found that 14% of these patients harbored enteric rods and pseudomonads. *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, and *Enterobacter agglomerans* comprised more than 50% of the strains isolated. Systemically administered ciprofloxacin improved the treatment response of patients whose periodontal pockets were heavily infected with enteric rods (Slots *et al*. 1990a). This group of investigators also examined 24 subjects with periodontal disease in the Dominican Republic and found that the prevalence of enteric rods in these subjects was higher than levels found in subjects in the US (Slots *et al*. 1991). In the 16 of 24 subjects in whom this group of organisms was detected, they averaged 23% of the cultivable microbiota. Rams *et al*. (1990, 1992) also identified a number of species of staphylococci and enterococci in subjects with various forms of periodontal disease. The presence of unusual species in periodontal lesions suggests the possibility that they may play a role in the etiology of periodontal diseases. However, such roles must be evaluated in the same manner as the species discussed earlier in this section.

In addition to the cultivable and uncultivable bacterial species, a number of studies have suggested that specific viruses, including cytomegalovirus, the Epstein Barr virus, papillomavirus, and herpes simplex virus, may play a role in the etiology or progression of periodontal lesions, possibly by changing the host response to the local subgingival microbiota (for a comprehensive review, see Slots 2005). A suspected role of various viruses was based primarily on association of the viruses with lesion sites when compared with periodontally healthy sites and the effect of successful therapy on reducing the detection frequency of these viruses in treated sites (Klemenc *et al*. 2005; Slots 2005; Slots *et al*. 2006).

Mixed infections

To this point, attention has been paid to the possible role of individual species as risk factors for destructive periodontal diseases. However, the complex mixture of species colonizing the subgingival area can provide a spectrum of relationships with the host, ranging from beneficial (the organisms prevent disease), to harmful (the organisms cause disease). At the pathogenic end of the spectrum, it is conceivable that different relationships exist between pathogens. The presence of two pathogens at a site could have no effect or could diminish the potential pathogenicity of one or the other of the species. Alternatively, pathogenicity could be enhanced either in an additive or synergistic fashion. It seems likely that mixed infections occur in subgingival sites since so many diverse species inhabit this habitat. Evidence to support this concept has been derived mainly from studies in animals in which it was shown that combinations of species were capable of inducing

experimental abscesses, even though the components of the mixtures could not (Smith 1930; Proske & Sayers 1934; Cobe 1948; Rosebury *et al*. 1950; Macdonald *et al*. 1956; Socransky & Gibbons 1965). It is not clear whether the combinations suggested in the experimental abscess studies are pertinent to human periodontal diseases. Studies in humans suggest that combinations of *P. gingivalis* and *T. forsythia* may be signifi cant in determining diseased sites and disease progression after treatment (Fujise *et al*. 2002). In addition, it has been observed that species such as *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans* may be components of a polymicrobial intracellular microbiota within human buccal epithelial cells (Rudney *et al*. 2005). At the very least, some species may set the stage for specifi c pathogens by providing essential nutrients, sites of attachment (co-aggregation), or means to evade or subvert host defenses (e.g. by producing protective capsules or enzymes that destroy host antibody). The relationship of microbial "complexes" to periodontal diseases will be discussed further below.

The nature of dental plaque – the biofilm way of life

Biofi lms colonize a widely diverse set of moist surfaces, including the oral cavity, the bottom of boats and docks, the inside of pipes, as well as rocks in streams. Infectious disease investigators are interested in biofi lms that colonize a wide array of artificial devices that have been implanted in the human, including catheters, hip and voice prostheses, and contact lenses. Biofi lms consist of one or more communities of microorganisms, embedded in a glycocalyx, that are attached to a solid surface. The biofilm allows microorganisms to stick to and multiply on surfaces. Thus, attached bacteria (sessile) growing in a biofi lm display a wide range of characteristics that provide a number of advantages over single cell (planktonic) bacteria. The interactions among bacterial species living in biofi lms take place at several levels including physical contact, metabolic exchange, small signal molecule mediated communication, and exchange of genetic information (Kolenbrander *et al*. 2006). References to pertinent biofi lm literature may be found in the following publications: Newman & Wilson (1999); Socransky & Haffajee (2001); Marsh (2005).