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Tannerella forsythia, a periodontal pathogen entering the genomic era

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Several questions need to be addressed to evaluate whether *Tannerella forsythia* is to be considered a periodontal pathogen. *T. forsythia* has been detected in periodontal health and disease, so could it be a pathogen? The species was not detected in many studies despite finding other putative pathogens, so could it be important in pathogenicity? The challenges of working with *T. forsythia* include its fastidious and anaerobic growth requirements for cultural detection. Thus, studies associating *T. forsythia* with periodontal and other oral infections have used noncultural approaches (immunoassays and DNA-based assays) in addition to cultural approaches. We feel the timing of this review represents an interesting transition period in our understanding of the relationships of species with infection. Information from the recently released full genome sequence data of *T. forsythia* will provide new approaches and tools that can be directed to assess pathogenicity. Furthermore, molecular assessment of gene expression will provide a new understanding of the pathogenical potential of the species, and its effect on the host.

T. forsythia, was described in reviews focusing on periodontal pathogens associated with herpesvirus detection (200), species for which genome projects were underway (41), members of polybacterial periodontal pathogenic consortium (91), and participants in periodontal microbial ecology (202). We will describe the history, taxonomy, and

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characteristics of *T. forsythia*, and related species or phylotypes in the genus *Tannerella*. To assess the pathogenic potential of *T. forsythia*, we first describe species associations with periodontal and other infections, including animal models, as has been the traditional approach arising from Koch's postulates (203). Criteria for pathogenicity were expanded to incorporate sequence- derived information (58), and again more recently to include molecular signatures of pathogens and disease (170). We used sequence and genome-derived information, in addition to biofilm, pathogenic mediators, and host responses, to further explore the pathogenic potential of *T. forsythia*.

History and taxonomy

T. forsythia was first isolated at The Forsyth Institute from subjects with progressing advanced periodontitis in the mid-1970s and was described as "fusiform *Bacteroides*" by Tanner et al. (225). Around the same time, *T. forsythia* was isolated as one of the *Bacteroides* group from the extensive cultural studies of periodontal infections by Moore and Holdeman- Moore at the Anaerobe Laboratory of the Virginia Polytechnic Institute (VPI). The species was subsequently detected by culture from oral samples at the Forsyth and VPI laboratories from progressing periodontitis (43, 153, 229), endodontic infections (232), gingivitis and early periodontitis (151, 221), refractory periodontitis (81), and peri-implantitis (223).

T. forsythia was initially a taxonomic enigma because it did not resemble described species of oral or enteric gram-negative anaerobic rods, particularly in its cell morphology and slow and fastidious growth requirements. As a gram-negative anaerobic rod with tapered ends, it was described as "fusiform *Bacteroides*" in our cultural report of progressing advanced periodontitis (225). Sufficient cells for characterization were obtained by enhancing growth by culturing *T. forsythia* on blood agar adjacent to *Fusobacterium nucleatum* cells. Growth stimulation by a number of species was observed during an extensive search to improve growth of the species (42). The moles percent (mol%) DNA content was determined to be around 46%, and is currently reported at 46.8% based on genome sequence (http://www.oralgen.lanl.gov). Strains collected from subgingival and endodontic lesions showed over 75–100% DNA

homology with each other. DNA homology with enteric *Bacteroides* species was for 0–31% *Bacteroides fragilis*, 0–28% for *Bacteroides thetaiotaomicron*, 0–20% for *Bacteroides vulgatus*, and 0–48% for *Bacteroides distasonis*. DNA homology with other oral "*Bacteroides*" (now *Prevotella*) species, was 0–30% for *Prevotella oris*, 5–14% for *Prevotella oralis*, and 11–17% for *Prevotella buccae*.

The phylogeny of oral *Bacteroides* species in the *Cytophaga–Flavobacterium–Bacteroides* family was reorganized after *Bacteroides forsythus* was described. Most oral "*Bacteroides*" species were reclassified to either *Porphyromonas* (188) or *Prevotella* (189), conserving the genus *Bacteroides* for the *B. fragilis*-like enteric species. Several species including *B. forsythus* and *Bacteroides distasonis*, did not belong in *Porphyromonas*, *Prevotella* or *Bacteroides.* These relationships were clarified in the phylogenetic studies comparing 16S rRNA sequence data by Paster et al. (168). These data confirmed the separation of oral gram-negative anaerobic rod species from enteric species. In the 16S rRNA sequence analysis (168), *B. forsythus* grouped with *B. distasonis*, consistent with the previous DNA homology data, but these two species clustered outside the *Prevotella* and *Bacteroides* groups. *B. forsythus* clustered most closely with *Porphyromonas* species, but not closely enough to be considered as *Porphyromonas*. The 16S rRNA phylogenetic analysis supporting reclassification of *B. forsythus* to the genus *Tannerella* further expanded the range of *Porphyromonas*, *Bacteroides*, and *Prevotella* species examined (179). The genus name was chosen for Anne Tanner who described the species as *B. forsythus* in 1986 (232). The Sakamoto 16S rRNA phylogenetic analysis confirmed the separation of *T. forsythia*, *B. distasonis*, and *Bacteroides merdae* (the latter both enteric species) from the other genera (179). The species was first formally reclassified to *Tannerella forsythensis*, then reclassified to *T. forsythia* (143) following a proposal to change the specific epithet (Brian J. Tindall 2005, personal communication). This specific epithet change was based on The Forsyth Institute being named for the Forsyth brothers, James, Thomas, and John, along with their sister Mary who founded the institution in 1910 as The Forsyth Dental Infirmary for Children. The specific epithet honors these benefactors.

The current taxonomic position of the genus *Tannerella* and related genera within the phylum *Bacteroidetes* based on 16S rRNA phylogenetic analysis is illustrated in **Fig. 1**. In particular, this figure illustrates the

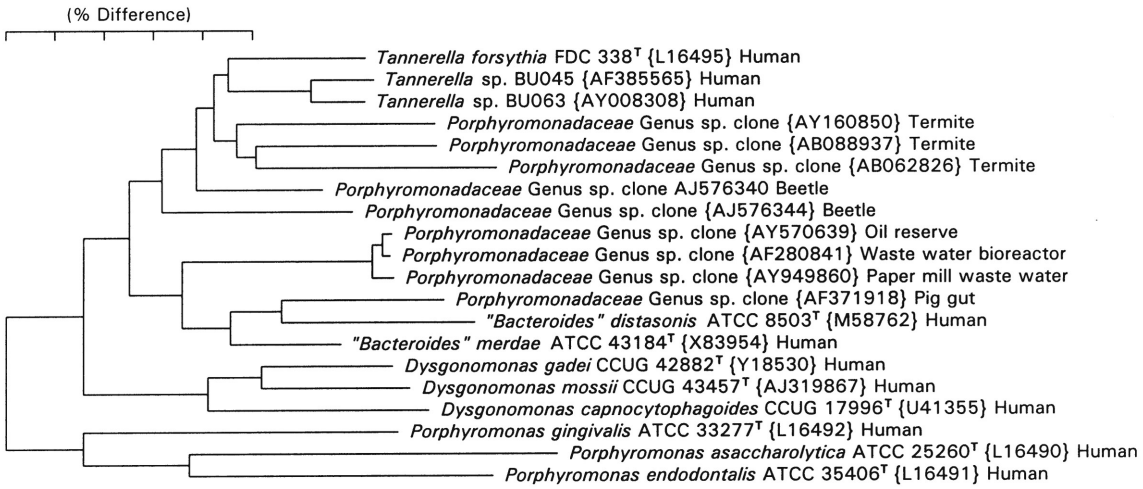

Fig. 1. Phylogenetic tree of the genus *Tannerella* and other genera in the phylum Bacteroidetes. The tree was constructed from aligned sequences using the neighbor-joining method. The naming scheme is as follows: (1) for cultivated species the official or provisional name was used; for not yet cultivated taxa the designation was to the closest genus or family (e.g. Porphyromonadaceae); (2) the 16S rRNA gene accession number is in curly brackets; and (3) original sample/isolate source. The bar denotes a 5% difference in sequence measured by summing the lengths of the horizontal lines between taxa.

phylogenetic relationship between *T. forsythia*, *B. distasonis*, and *B. merdae*. Three *Porphyromonas* species are also included to illustrate their relationship with *Tannerella*.

T. forsythia from non-human hosts and other Tannerella phylotypes

Strains of *T. forsythia* have been isolated from the subgingival plaque of monkeys, and in humans from cat and dog bite wounds (**Fig. 2**). The *T. forsythia* strains isolated from monkeys were quite similar to the *T. forsythia* strains from humans both biochemically and in their requirement for *N*-acetylmuramic acid (12). Comparison of human and monkey isolates with those from cat and dog bite wounds indicated that dog and cat bite isolates differed in their lack of requirement for *N*-acetylmuramic acid for growth, and catalase activity. In addition, cat and dog bite isolates differed by their phenylalanine aminopeptidase activity compared to the monkey isolates (94). Previous phylogenetic analysis of 16S rRNA gene sequences indicated that the monkey isolates were more similar

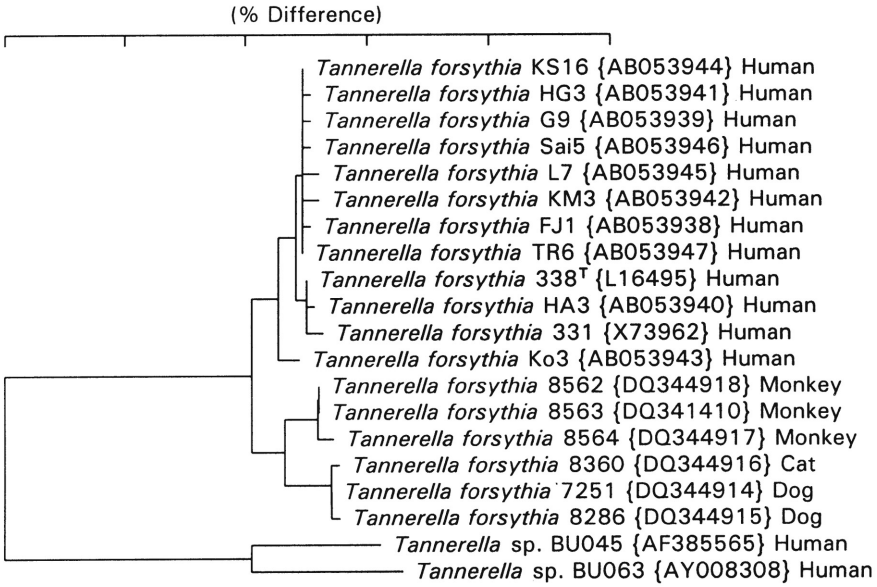

Fig. 2. *Tannerella forsythia* 16S rRNA distribution based on sequences deposited in GenBank. The tree was constructed from aligned sequences using the neighbor-joining method. The naming scheme is as follow: (1) official or provisional names; (2) the 16S rRNA gene accession number in curly brackets; and (3) original sample/isolate source. The bar denotes a 5% difference in sequence as measured by summing the lengths of the horizontal lines between taxa. The 16S rRNA gene sequences from monkey oral isolate (12), as well as dog and cat bite (94) have been deposited in GenBank for the purpose of this review under the accession numbers DQ341410, and DQ344914–DQ344918.

to the type strain from human, than to the dog and cat isolates (94). The new phylogenetic analysis in Fig. 2 illustrates that the monkey isolates and strains from dog and cat bite wounds are closely related to human isolates of *T. forsythia* and all belong in the same species.

Three distinct *Tannerella* phylotypes have been recognized from human subgingival sites. In addition to *T. forsythia*, two other phylotypes, BU063 and BU045, were identified from a clone library (167). The phylogenetic trees of Fig 1 and 2 illustrate that the oral clones BU045 and BU063 fall within the genus *Tannerella* but not within the *T. forsythia* species. There are currently no cultured isolates with sequences similar to those of the BU045 and BU063 clones. Sequence-based polymerase chain reaction methods indicated that *T. forsythia* was detected more frequently from periodontitis, relative to the *Tannerella* BU063 clone phylotype which was detected more frequently from periodontal health

(132). In the latter study, DNA was first amplified from plaque samples using universal (broad range) primers for regions in 16S and 23S genes. A second, nested, polymerase chain reaction amplification included a sequence in the 23S rDNA gene, thus amplifying the intergenic spacer region. This analysis yielded product/amplicons at different molecular weights, corresponding to the different phylotypes (132).

Examination of 16S rRNA sequence data deposited in GenBank indicates that several other *Tannerella* organisms have been detected in the soil and from the gut of insects (Fig. 1). These microorganisms emerged from polymerase chain reaction-cloning sequencing analyses, and only clone sequences are available, not cultured isolates. These phylotypes were detected in the guts of soil-feeding termites (186), scarab beetle larvae (47), and wood-eating termites (194). These *Tannerella* species may co-exist with *Treponema* species that have recently been cultured with unusual and fastidious growth requirements from termite guts (70). The environment for these insect-isolated *Tannerella* and *Treponema* organisms is clearly different from that of the human oral cavity, with markedly different nutrient sources. Detection of phylotypes of genera in oral and environmental samples has also been reported for uncultivated TM7 (96, 167), Obsidian pool (96, 167), and *Archea* (131). One might speculate that soil organisms were the original source for these fastidious species and that the growth requirements of *Tannerella* and uncultivated phylotypes may reflect the original source of the species.

Characteristics of *T. forsythia* and identification in clinical samples

Characteristics of isolates

The growth of *T. forsythia* is stimulated by *N*-acetylmuramic acid (260). The shape of *T. forsythia* cells and colonies varies depending on the growth conditions. Cells of cultures grown on agar media without *N*acetylmuramic acid are large, filamentous, and pleomorphic, with tapered (fusiform) ends, also with spheroids. As described above, the growth requirement for *N*-acetylmuramic acid was observed for strains isolated from dental plaque from humans and monkeys (*Macaca fascicularis*) (12), but not for the *T. forsythia* strains isolated from dog and cat bite

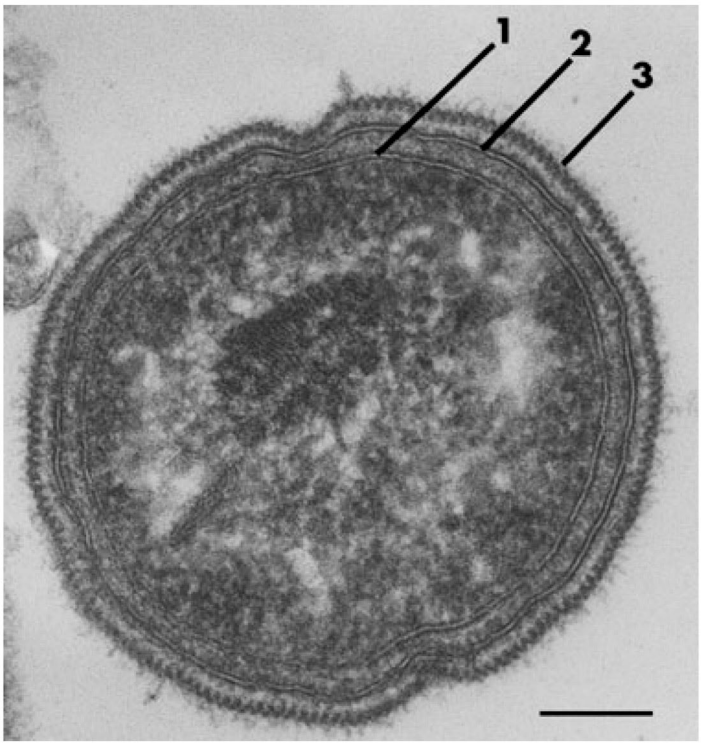

Fig. 3. Cell cross-section of *Tannerella forsythia* ATCC 43037. The cells were fixed in sodium cacodylate buffer, dehydrated, and embedded in Epon. Sections were mounted on grids and stained with uranyl acetate and lead citrate (232). (1) Inner-membrane, (2) Outer-membrane, and (3) Distinctive outer layer. Scale bar = 0.1 μm. Reprinted with the permission of the *International Journal of Systemic and Evolutionary Microbiology.*

wounds (94). *T. forsythia* has a distinctive ultrastructure (**Fig. 3**). Below a distinctive outer layer (S layer) there is an inner membrane, and an outer membrane (232). Colonies are tiny and opaque. Colony morphology changes in the presence of either *N*-acetylmuramic acid or a growth-stimulating species, for example *F. nucleatum* (232). Colonies become pale pink and speckled, circular, entire, slightly convex, and may have a depressed center (donut-shaped). In the presence of *N*-acetylmuramic acid, cells become regularly shaped, short, gram-negative rods (12).

Biochemically *T. forsythia* can be deceptively inert when tested using slight broth growth achieved in the absence of *N*-acetylmuramic acid. When cells are harvested from agar the biochemical reactivity of the species becomes apparent. Biochemical reactivity from resting cell tests (enzyme substrate tests) has been performed on commercially available strips (179, 230) and from chromogenic reagents (145). *T. forsythia*

has the ability to metabolize a range of substrates and, in common with many enteric *Bacteroides* species, to hydrolyze esculin. In contrast to the enteric species, however, *T. forsythia* is not resistant to bile, thus it does not grow on the routine *Bacteroides* medium for enteric species, Bile Esculin Agar (179). Because of slight broth growth of *T. forsythia* when originally described, characterization included whole cell protein profiles on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which for isolates of human origin demonstrated a signature double band greater than 200 kDA (232). All strains tested, except one monkey isolate (94), also demonstrated these protein bands, which have been characterized as S-layer bands (90).

Cultural isolation of periodontal species, particularly the nutritionally fastidious pathogens of the "red complex" (205), requires more than including all the various growth-stimulating factors in a single medium. Isolation and cultivation of oral treponemes, including *Treponema denticola*, is routinely performed in specialized media (104, 241), which is commercially available (Anaerobe Systems, Morgan Hill, CA). While *Porphyromonas gingivalis* and *T. forsythia* will grow on media supplemented with vitamin K (menadione) for *P. gingivalis*, and *N*-acetylmuramic acid for *T. forsythia*, each species is inhibited by the growth requirement of the other, so compromising primary isolation (226). Both *T. forsythia* and *P. gingivalis* are anaerobes but it also has been suggested that they may, similar to *B. fragilis* (9), be able to grow in the presence of low levels of oxygen. This has been demonstrated for *P. gingivalis* (36). On primary isolation from oral samples, however, strict anaerobiosis favors isolation of *T. forsythia* and other subgingival anaerobes (39).

Antibiotic sensitivity

T. forsythia is generally sensitive to antibiotics that are active against anaerobes. Most active antibiotics were amoxicillin with clavulanate (100%), ampicillin (98%), doxycycline (98%), amoxocillin (96%), tetracycline (90%), and clindamycin (86%) (126). Fairly active antibiotics were penicillin (70%) and spiramycin (68%) whereas poorly active antibiotics were erythromycin (54%) and ciprofloxacin (46%) (126). In addition, *T. forsythia* was found to be sensitive to metronidazole (216). Results from a study of Brazilian isolates provided similar observations

to these studies (139). Sensitivity to tetracycline is consistent with the failure to detect *T. forsythia*-resistant strains after local tetracycline therapy (67). Resistance to penicillin can be mediated by the production of β-lactamase. Isolates of *T. forsythia* with β-lactamase activity were identified from two of 23 (8%) untreated adult periodontitis subjects (11% two of 19 total isolates) (246). Addition of clavulanic acid to amoxicillin overcomes β-lactamase inactivation of penicillin, as described in a recent review (253).

The genome sequence of *T. forsythia* ATCC 43037 (see below) includes a copy of *tet*(Q), a gene encoding the ribosome protection protein resulting in antibiotic resistance to tetracycline. This observation adds *T. forsythia* to the list of oral organisms with this mechanism of antibiotic resistance (251). Not all genomes from oral isolates encode for a ribosome protection protein, like *tet*(Q), or an efflux pump providing antibiotic resistance to the organism (251). In addition to *tet*(Q) genes, strains of *T. forsythia* contained *erm*(F) which codes for erythromycin resistance (244).

Other antibiotic, or antimicrobial formulations with activity against *T. forsythia* include flurithromycin (134), moxifloxacin (149), and fanopenem (148). *T. forsythia* is sensitive to several non-antibiotic, antibacterial approaches which are designed for topical or local administration. These include silver nitrate (210), and chlorin e6 conjugated to pentalysine-5K in conjunction with laser photodynamic therapy (172). Topical activity of chlorhexidine gluconate (PerioChip) against *T. forsythia* provided little suppression compared with scaling and root planing (28). Good activity *in vitro* was demonstrated using a different polymer-based vehicle (266).

Identification from clinical samples

A number of methods have been used to detect *T. forsythia* in clinical samples and host tissues. Detection methods included immunoassay and genetic/molecular assays. In this review we focus on methods used to identify *T. forsythia* in samples rather than providing a comprehensive review of microbiological identification methods, for which there are already some excellent reviews (23, 161, 181, 184, 196, 202).

Cultural identification

Identification tests when using cultural analysis were simplified from the range of tests used to characterize the species for classification and taxonomy. Slots presented a scheme for rapid identification of *P. gingivalis* (then *Bacteroides gingivalis*) from culture (199) comprising colony pigmentation, lack of fluorescence by long-wave UV light, and negative lactose fermentation (MUG test). This was adapted to *T. forsythia*, which differs in colony morphology and positive lactose fermentation (MUG test). Other strain identification schemes incorporate characterization using, for example, chromogenic test strips from API, or fluorogenic enzyme substrate tests (145). Key tests include α-glucosidase, β-glucosidase, sialidase, and negative indole in addition to positive trypsin-like activity (12). In a comparison of culture with a DNA-probe assay, definitive identification of *T. forsythia* was obtained by a combination of colony morphology, trypsin-like activity and sodium dodecyl sulfate–polyacrylamide gel electrophoresis for a specific identification (226).

The ability of *T. forsythia* to hydrolyze the trypsinlike benzyol-DL-arginine-2-naphthylamide, "BANA", has been incorporated in the test for periodontal pathogens pioneered by Loesche (136). Trypsin-like activity is shared by *P. gingivalis* and *T. denticola* of the Socransky "red complex" (205). Other subgingival species are also BANA-positive, but not as strongly as *P. gingivalis*, *T. denticola*, and *T. forsythia*, thus making this biochemical marker a test for the presence of these species. The BANA test in the U.S.A., or SK-013 test in Japan, was devised for rapid clinical detection of a periodontal pathogen-containing microbiota (137, 187).

Immunological assays

Antibody raised against *T. forsythia* cells and used for species identification, did not cross-react with *P. gingivalis*, *Prevotella intermedia*, or *Prevotella melaninogenica* (44). Antibody to *T. forsythia* was serologically distinct from other oral and clinical "*Bacteroides*" species (now *Bacteroides*, *Porphyromonas*, and *Prevotella*) (45). Lai et al. (125) demonstrated that polyclonal antibody to *T. forsythia* in an indirect immunofluorescence assay did not react with an extended range of oral gram-negative and gram-positive species. Monoclonal antibodies were also produced against *T. forsythia* that were strain and species specific and

that did not react with other subgingival species (255). They were subsequently used in enzyme-linked immunosorbent assays and immunofluorescence assays (255). Immunoassays have been used extensively by several groups of investigators for rapid identification of *T. forsythia* in periodontal health and diseases (**Table 1**). Direct identification from clinical samples bypasses the challenges of species culture. Lai et al. in Philadelphia U.S.A. (125) used polyclonal antibody in an immunofluorescence assay, and demonstrated change in detection after therapy. Gmu¨ r et al., in Zurich Switzerland, used specific monoclonal antibodies in immunofluorescence assays to evaluate associations of *T. forsythia* with periodontal health and disease (63–65). Zambon et al. incorporated immunofluorescence assay identification of *T. forsythia* to evaluate microbial sampling protocols (75) and in studies of risk indicators for periodontal disease (72, 73). Immunofluorescence assay to identify *T. forsythia* was also used in our comparison of rapid (chair-side) DNA probe identification with culture to clarify the status of culture-negative, probe-positive samples (226).

Molecular identification

Molecular methods for species identification in samples have evolved from DNA/DNA homology to sequence-based methods used in microbial taxonomy (167, 179, 224, 232). The diversity of the oral species based on molecular methods used the comprehensive polymerase chain reaction/cloning/ sequencing steps (124, 157, 167, 182). Rapid identification of *T. forsythia* using molecular methods may be broadly grouped into DNA probe and polymerase chain reaction methods, although several approaches incorporate both technologies.

DNA probes

DNA probe methods have been used in conjunction with culture or directly from oral samples. DNA probes have been made from whole genomic DNA, from species-specific cloned DNA fragments or oligonucleotide sequences. Whole genomic probes for *T. forsythia* showed minimal cross-reactivity with other species (202, 208). The colony-lift method used colonies from primary isolation plates that had been transferred (lifted) onto nylon membranes (74). Using primary isolation plates

Table 1. Evidence for association of *Tannerella forsythia* with periodontal disease

Study	*Bacterial identification method*	*Disease category*	*Study design*
Association with moderate to advanced periodontitis			
Tanner et al. (1979) (225)	Cultivationa	Chronic periodontitis, progressing sites	Cross-sectional
Tanner et al. (1984) (229)	Cultivation[a]	Chronic periodontitis, progressing sites	Longitudinal
Dzink et al. (1985) (43)	Cultivation[a]	Chronic periodontitis, progressing sites	Longitudinal
Lai et al. (1987) (125)	Fluorescent antibody	Chronic periodontitis	Cross-sectional
Moore et al. (1987) (152)	Cultivation	Chronic periodontitis	Cross-sectional
Haffajee et al. (1988) (82)	Culture	Chronic periodontitis, progressing sites	Longitudinal
Moore et al. (1991) (153)	Cultivation	Chronic periodontitis, progressing sites	Longitudinal
Christersson (1992) (21)	Indirect immunofluorescence (IFA)	Chronic periodontitis	Cross-sectional
Haffajee et al. (1992) (83)	Whole genomic hybridization from cultivated colonies	Chronic periodontitis, progressing sites	Cross-sectional
Haffajee et al. (1994) (78)	Cultivation, whole genomic hybridization	Chronic periodontitis	(Review)
Moore et al. (1994) (154)	Cultivation	Chronic periodontitis	(Review)
Chan et al. (1994) (16)	Immunofluorescence	Chronic periodontitis (Taiwan)	Cross-sectional
Grossi et al. (1994) (73)	Immunofluorescence	Chronic periodontitis	Cross-sectional
Kamma et al. (1994) (113)	Cultivation	Aggressive periodontitis (Greece)	Cross-sectional
Grossi et al. (1995) (72)	Immunofluorescence	Chronic periodontitis	Cross-sectional
Kamma et al. (1995) (114)	Cultivation	Aggressive periodontitis (young adults, Greece)	Cross-sectional
Ali et al. (1997) (2)	Whole genomic hybridization	Chronic periodontitis (Northern Cameroon)	Cross-sectional
Di Murro et al. (1997) (35)	ELISA antibody	Chronic periodontitis	Cross-sectional
Machtei et al. (1997) (141)	Immunofluorescence	Chronic periodontitis (Attachment loss prediction)	Longitudinal
Papapanou et al. (1997) (163)	Whole genomic hybridization	Chronic periodontitis, progressing sites	Longitudinal
Dibart et al. (1998) (37)	Whole genomic hybridization	Chronic periodontitis, (in/on crevicular epithelial cells)	Longitudinal
Umeda et al. (1998) (237)	16S rRNA PCR	Chronic periodontitis, four racial/ethnic groups	Cross-sectional
Haffajee et al. (1998) (77)	Whole genomic hybridization	Chronic periodontitis	Cross-sectional
Genco et al. (1990) (60)	Immunofluorescence	Chronic periodontitis	Cross-sectional
Kamma et al. (1999)(112)	Cultivation	Aggressive periodontitis (in smokers)	Cross-sectional
Choi et al. (2000)(20)	PCR	Chronic periodontitis (Korea)	Cross-sectional
Darby et al. (2000)(29)	PCR	Aggressive and chronic periodontitis	Cross-sectional
Herrera et al. (2000)(89)	Cultivation	Periodontal abscess	Cross-sectional
Kasuga et al. (2000)(117)	PCR	Chronic periodontitis	Cross-sectional
Mullally et al. (2000)(156)	PCR	Aggressive periodontitis (Ireland)	Cross-sectional
Papapanou et al. (2000)(165)	Whole genomic hybridization	Chronic periodontitis	Cross-sectional
Ximenez-Fyvie et al. (2000)(261)	Whole genomic hybridization	Chronic periodontitis	Cross-sectional
Yano-Higuchi et al. (2000)(264)	Cultivation, DNA probe	Aggressive and chronic periodontitis (Japan)	Cross-sectional
Darby et al. (2001)(31)	PCR	Chronic periodontitis	Longitudinal
Trevilatto et al. (2002)(235)	PCR	Aggressive periodontitis (Brazil)	Cross-sectional

Continued

Table 1. Evidence for association of *Tannerella forsythia* with periodontal disease (*continued*)

Study	*Bacterial identification method*	*Disease category*	*Study design*
Leys et al. (2002)(132)	PCR	Chronic periodontitis	Cross-sectional
van Winkelhoff et al. (2000)(245)	Cultivation	Chronic periodontitis	Cross-sectional
Colombo et al. (2002)(22)	Whole genomic hybridization	Chronic periodontitis (Brazil)	Cross-sectional
vila-Campos et al. (2002)(250)	Cultivation	Chronic periodontitis (Brazil)	Cross-sectional
Darout et al. (2003)(32)	Whole genomic hybridization	Chronic periodontitis (Sudan)	Cross-sectional
Huang et al. (2003)(93)	AP-PCR	Chronic and refractory periodontitis (different genotypes)	Cross-sectional
Kumar et al. (2003)(123)	PCR	Chronic periodontitis	Cross-sectional
Dogan et al. (2003)(40)	Cultivation/PCR	Chronic periodontitis (Turkey)	Cross-sectional
Klein et al. (2003)(119)	PCR	Chronic periodontitis	Cross-sectional
Lee et al. (2003)(128)	PCR/Oligonucleotide Probe	Aggressive periodontitis (Korea)	Cross-sectional
Takeuchi et al. (2003)(217)	PCR	Aggressive periodontitis	Cross-sectional
Yang et al. (2004)(263)	Immunofluorescence	Chronic and aggressive periodontitis	Cross-sectional
Kumar et al. (2004)(124)	16S RNA clone, sequence	Chronic periodontitis	Cross-sectional
Campus et al. (2005)(14)	PCR (Multiplex)	Chronic periodontitis, Diabetes	Cross-section[a]
Association with progressing periodontitis post therapy (refractory periodontitis)			
Lai et al. (1987)(125)	Fluorescent antibody	Chronic periodontitis	Cross-sectional
Haffajee et al. (1988)(81)	Cultivation	Chronic periodontitis	Longitudinal
Shiloah et al. (1988)(193)	DNA Probes	Chronic periodontitis	Longitudinal
Socransky et al. (1993)(204)	Whole genomic hybridization	Chronic periodontitis	Longitudinal
Winkel et al. (1997)(256)	Cultivation	Chronic periodontitis	Cross-sectional
Edwardsson et al. (1999)(46)	Cultivation	Chronic periodontitis	Longitudinal
Lo Bue et al. (1999)(135)	Cultivation	Chronic periodontitis	Cross-sectional
Socransky et al. (2002)(207)	Whole genomic hybridization	Chronic periodontitis	Longitudinal
Huang et al. (2003)(93)	AP-PCR	Chronic periodontitis (different genotypes)	Cross-sectional
Heitz-Mayfield et al. (2006)(88)	Whole genomic hybridization	Chronic periodontitis	Longitudinal
Association with early periodontitis			
Tanner et al. (1989)(221)	Cultivation	Chronic periodontitis, progressing sites	Cross-sectional
Tanner et al. (1996)(222)	Cultivation	Chronic periodontitis, progressing sites	Longitudinal
Tanner et al. (1998)(231)	Cultivation, Whole genomic hybridization	Chronic periodontitis, progressing sites	Longitudinal
Machtei et al. (1999)(142)	Immunofluorescence	Chronic periodontitis, (disease prediction)	Longitudinal
Tran et al. (2001)(234)	PCR (Multiplex)	Chronic periodontitis, progressing sites	Longitudinal
Hamlet et al. (2004)(84)	PCR	Chronic periodontitis, progressing sites (adolescents)	Longitudinal
Suda et al. (2004)(211)	Immunofluorescence	Chronic periodontitis (adolescents)	Cross-sectional
Tanner et al. (2006)(228)	PCR (Multiplex)	Chronic periodontitis	Cross-sectional

a. *Tannerella forsythia* identified as "fusiform" *Bacteroides*

allowed species amplification by assaying colonies of isolates rather than cells in plaque samples (74). Direct analysis of clinical samples using whole genomic DNA probes for *T. forsythia* had a sensitivity of 10^3 to 10^4 cells (138). Use of whole genomic probes was expanded by Socransky et al. (208) to analyze several species against multiple samples simultaneously using a "checkerboard" assay. This method can assay subgingival samples ranging in size from 10^4 to 10^7 cells in a sample. Larger samples can be analyzed by diluting samples, thus avoiding cross-reactivity between species analyzed using whole-genomic probes. A combination of culture and whole genomic checkerboard analysis was developed to assay antimicrobial susceptibility of subgingival samples (51) and artificial biofilms (209). In this assay all colonies from plates were harvested, the cells were diluted into the assay range, and tested against whole genomic probes in the checkerboard assay. Randomly cloned DNA probes to *T. forsythia* were more specific than whole genomic probes but less sensitive for species detection (258). Cloned probes are the basis of a commercial laboratory assay which is available in Europe (as DMDx/PATHOTEK®)s (23).

DNA probe methods also use oligonucleotide probes to identify *T. forsythia* and other species in oral and clinical samples. Oligonucleotide probe detection (38) of *T. forsythia* and other selected species were used in the evaluation of local tetracycline therapy for periodontitis (68). These oligonucleotide probes included several different oligonucleotide sequences complementary to species-specific 16S rRNA sequences to improve the sensitivity of species identification and quantification in clinical plaque samples. The reverse capture–oligonucleotide probe assay uses a similar checkerboard apparatus as that used in the whole genomic probe checkerboard assay. To use oligonucleotide probes, samples first undergo a polymerase chain reaction amplification using broad-range "universal" primers (166, 208). Microarrays or DNA chips represent a miniaturized version of assaying samples to multiple species using oligonucleotide DNA probes. One commercially available microarray, the ParoCheck® (Greiner, Bio-One GmbH, Frickenhausen, Germany) includes *T. forsythia* among the 20 assayed species.

Direct polymerase chain reaction methods

Detection of several putative periodontal pathogens including *T. forsythia* directly from subgingival samples using species-specific primers

in a polymerase chain reaction was described by Slots and co-workers (6, 201). Different primer sets have been described by a number of investigators to detect *T. forsythia* in periodontal samples (84, 119). Polymerase chain reaction methods were also used to detect *T. forsythia* in endodontic lesions (25). Multiplex polymerase chain reaction allows the detection of several species in a single polymerase chain reaction assay. Multiplex polymerase chain reaction assays including *T. forsythia* have been described (14, 15, 24, 233). Several recent reports describe the use of real-time polymerase chain reaction to quantify *T. forsythia* in samples (106, 122, 127, 180, 214).

Comparisons between identification methods

Culture and polymerase chain reaction-based methods

Cultural detection of *T. forsythia* is compromised if *N*-acetylmuramic acid is not included in the isolation medium and too short (<10 days) an incubation time is used for primary isolation. There are few comparisons between polymerase chain reaction followed by cloning and sequence-based identification and culture. One such comparison used five endodontic samples and reported that cultural techniques favored detection of *Bacteroidetes* over the polymerase chain reaction-based method. *T. forsythia* was detected in one subject by culture but not by the polymerase chain reactionbased method (157). This finding led to an improvement of the polymerase chain reaction primers, for *Bacteroidetes* (33) but no further comparisons with culture were reported.

Other studies comparing the cultural detection of *T. forsythia* with other methods, detected the species more frequently by non-cultural methods. Immunofluorescence assays detected *T. forsythia*, *P. gingivalis*, *P. intermedia*, and *Campylobacter rectus*, more frequently than by culture (62). Furthermore, comparisons between culture and rapid polymerase chain reaction-based methods for *T. forsythia* indicated increased detection frequency for the polymerase chain reaction methods. One study detected 21% culture positive compared with 27% positive in a multiplex polymerase chain reaction assay (49). Culture compared with real-time polymerase chain reaction indicated that all culture positive samples were detected by real-time polymerase chain reaction, but that culture failed to detect a further 28% of samples detected only by polymerase chain reaction (106). A more marked difference between culture and

real-time polymerase chain reaction was reported by Lau et al. (127), with a detection of *T. forsythia* of 12/92 by culture and 82/92 by real time polymerase chain reaction, which detected all the culture positive samples.

Culture and DNA probes

A comparison between culture (with *N*-acetylmuramic acid in primary isolation plates) and whole genomic probes in the checkerboard assay also detected *T. forsythia* more frequently by the genetic assay than by culture (164). The *T. forsythia* was detected by both methods in 27% of samples, and by culture or probe in 11.5% and 61.5% of samples, respectively (164). An oligonucleotide probe assay comprising two probe sequences also detected *T. forsythia* more frequently than by culture (150). In that study 18/20 samples were probe-positive compared with 13/20 culture-positive. These authors observed that in probe-positive, culture-negative samples, *T. forsythia* was detected in very low proportions (<1%) of the total microbiota (150), which was below the detection threshold for non-selective culture (150). Similar findings were reported using a similar composite probe to the latter report but used in a rapid chair-side assay (226, 236). Ali et al. (3) compared data from the same chair-side probe assay with culture data and detected a much higher proportion of *T. forsythia*-positive samples by probe (15/54) compared with anaerobic culture (3/54). In a second study that included whole genomic probe checkerboard assay, it was found that 28% and 18% of the samples were positive by probe and culture, respectively (2). The checkerboard assay, however, detected a higher proportion of positive samples (81%) than the oligonucleotide test or culture (2). The Paro-Check® (Greiner, Bio-One GmbH, Frickenhausen, Germany) microarray was more sensitive for *T. forsythia* than culture. *T. forsythia* was detected in eight of 20 root canals using the probe assay but was not detected by culture (249).

Other comparison studies did not include culture. A *T. forsythia* oligonucleotide probe, complementary to single 16S rRNA sequence, demonstrated good agreement with an immunofluorescence assay, suggesting similar sensitivities between the two assays for the detection of subgingival species (61). The whole genomic checkerboard assay and polymerase chain reaction for *T. forsythia* gave similar detection frequencies

(197). Comparisons between end point and quantitative (real-time) polymerase chain reaction with an enzyme-linked immunosorbent assay indicated that the polymerase chain reaction assays were more sensitive than enzyme-linked immunosorbent assay (192).

Collectively these comparisons indicate that *T. forsythia* can be detected reliably and sensitively in samples using non-cultural methods. Use of oligonucleotide probes and polymerase chain reaction- based studies has the advantage of being less susceptible to cross-reactivity than whole genomic probe assays. Sequence-based methods, oligonucleotide probes and polymerase chain reaction have a further advantage in being able to assay cultured species and phylotypes for which there are currently no cultivated microorganisms, including *Tannerella* phylotypes (Fig 1 and 2).

Association with oral infections

Association with periodontal infections

Association with disease encompasses more frequent and/or higher levels of suspected pathogen detection in disease compared with health, and species suppression (elimination) with resolution of symptoms (58, 203). *T. forsythia* was first recognized from advanced periodontitis in subjects who had shown recent disease progression based on serial radiographs (225). The association of *T. forsythia* with periodontitis, including progressing (aggressive) periodontitis, has been observed in a number of studies from populations around the world (Table 1). Periodontitis that progresses post-therapy, "refractory " periodontitis, represents a particularly aggressive form of disease, which has also been associated with detection of *T. forsythia* (Table 1). Detection methods for the species include cultural, immunological, and genetic assays.

T. forsythia has also been associated with early or initial periodontitis (Table 1). It had been hypothesized that one set of species might initiate gingivitis, after which other species would initiate periodontitis. Detection of periodontal pathogens in gingivitis and early periodontitis suggests that similar species initiate both infections. *T. forsythia* has been associated with early stages of periodontal attachment loss by culture (231), immunofluorescence (142), and polymerase chain reaction (234).

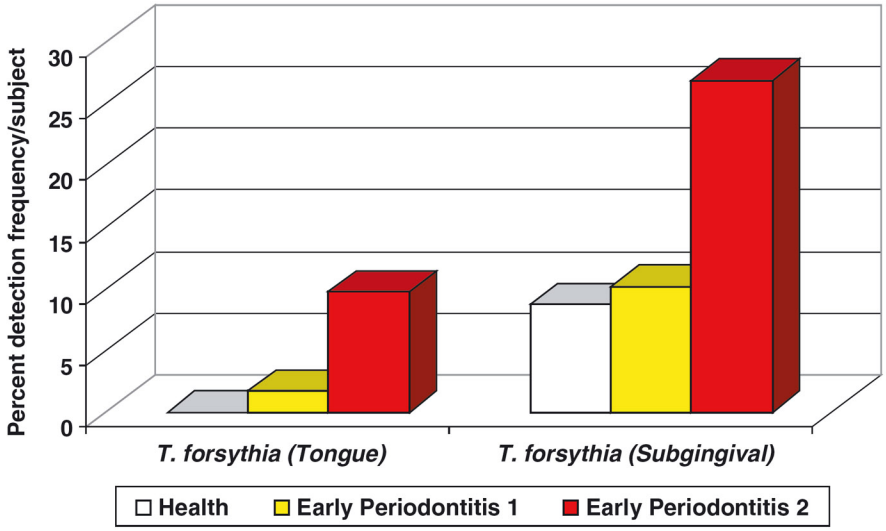

Fig. 4. Detection by PCR of *Tannerella forsythia* in 124 paired tongue and subgingival samples of 23 healthy (mean periodontal attachment level (AL) ≤1.5 mm, no sites >2 mm AL loss), 59 early periodontitis 1 (mean AL ≤1.5 mm, ≥1 site more than 2 mm AL loss) and 42 early periodontitis 2 (mean AL >1.5 mm) subjects. *T. forsythia* was detected more frequently subgingivally than from tongue samples (P = 0.001). *T. forsythia* in subgingival samples (P < 0.03) was associated with early periodontitis. Data derived from (228).

We recently examined early stages of periodontal attachment loss compared with periodontal health and compared the microbiota by polymerase chain reaction and 16S rRNAbased oligonucleotide probes (228). Detection of *T. forsythia* was associated with early periodontitis in a comparison of subgingival and tongue samples from healthy subjects and those with early periodontitis in this cross-sectional analysis (**Fig. 4**). Detection associated with bleeding on probing or attachment loss in adolescents has also been recorded (84, 211), further suggesting an association with early periodontitis.

Suppression of a pathogen with resolution of disease represents further evidence for disease association. Studies that observed a reduction of *T. forsythia* associated with resolution of clinical symptoms of disease are summarized in **Table 2**. A range of therapies was used to achieve clinical improvement including scaling and root planing, local and/or systemic antibiotics, and periodontal surgery.

Risk factors for periodontitis have also been linked with increased detection of *T. forsythia*. Detection of *T. forsythia* was associated with

Table 2. Studies linking suppression of *T. forsythia* post therapy with disease resolution

Study	*Bacterial identification method*	*Therapy type*	*Study design*
Haffajee et al. (1996)(80)	Whole genomic hybridization	Periodontal surgery, systemic tetracycline	Longitudinal
Umeda et al. (1996)(239)	Cultivation IFA	Local minocycline	Longitudinal
Haffajee et al. (1997)(76)	Whole genomic hybridization	Scaling, root planing (SRP)	Longitudinal
Winkel et al. (1997)(256)	Cultivation	SRP, systemic metronidazole	Longitudinal
Winkel et al. (1998)(257)	Cultivation	Systemic amoxicillin and metronidazole	Longitudinal
Takamatsu et al. (1999)(215)	DNA probe, PCR	SRP	Longitudinal
Cugini et al. (2000)(27)	Whole genomic hybridization	SRP	Longitudinal
Darby et al. (2001)(31)	PCR	SRP	Longitudinal
De Soete et al. (2001)(34)	Whole genomic hybridization	SRP, chlorhexidine (whole mouth disinfection)	Longitudinal
Feres et al. (2001)(52)	Whole genomic hybridization	Systemic amoxicillin or metronidazole	Longitudinal
Van der Velden et al. (2003)(240)	Cultivation	SRP +/) surgery, antibiotics	Longitudinal
Rodrigues et al. (2004)(171)	Cultivation, DNA probes	SRP, tetracycline (systemic or local)	Longitudinal
Darby et al. (2005)(30)	PCR	SRP	Longitudinal

subjects who were smokers (46, 79, 112, 242, 267), positive for aspartate aminotransferase activity (115), or interleukin-1 genotype (PST test) (206). Systemic disease is frequently associated with lowered resistance to infection, including periodontal infections. *T. forsythia* was associated with viral diseases (200), subjects infected with HIV (66, 146), diabetes, (14), and Papillon–Lefe`vre syndrome (140, 173).

Certain populations have an elevated risk of developing periodontitis, which might reflect differences in the subgingival microbiota. In the UK, *P. gingivalis* was detected more frequently in Indo- Pakistani adolescents compared with white-Caucasian and Afro-Caribbean adolescents (50). In Los Angeles (U.S.A.), *P. gingivalis* was also detected more frequently in advanced periodontitis in Asian patients compared with African-Americans and Caucasians, but the highest *P. gingivalis* detection frequencies were in Hispanic subjects, although these differences were not significant (237). In the same population, *T. forsythia* had similar detection frequencies between groups, *T. forsythia* was detected in 100% of Hispanic advanced periodontitis subjects. Craig et al. (26) reported lower serum immunoglobulin G levels to *T. forsythia* in Hispanic subjects. In the latter study, the differences in periodontitis, microbiota and host response were considered to be related more to environmental and demographic factors, including occupational status, than to race/ethnicity.

While *T. forsythia* has been associated with periodontitis, it was also detected in gingival and subgingival plaque in periodontally healthy subjects (63, 64). Detection in periodontal health may represent its presence

in a "carrier state", which seems likely because levels are generally lower than those found in periodontal disease (Table 1). Furthermore, different bio- or genotypes may have different relationships with health or disease. Different phylotypes of *Tannerella* were associated with periodontal health compared with *T. forsythia* in periodontitis (123, 132). Detection of different genotypes, as determined by reverse transcription-polymerase chain reaction (138) or arbitrarily-primed-polymerase chain reaction (93), showed different disease associations. *T. forsythia* has also been detected in samples from the tongue, and was associated with halitosis (219). In our studies of healthy and early periodontitis *T. forsythia* was detected more frequently subgingivally than from the tongue (Fig. 4) (228), suggesting that the likely primary niche/habitat is subgingival. This observation was similar to that of other periodontal species detected using oligonucleotide probes, but contrasted with *P. gingivalis* which was associated with both tongue and subgingival sites (228).

Association with endodontic and peri-implant oral infections

T. forsythia is not exclusively associated with periodontal infections. Strains used in the taxonomy study originated from both periodontal and endodontic lesions. Taxonomic studies have looked at *T. forsythia* isolates from peri-apical infection (105) and from alveolar radiolucent lesions of endodontic origin (17). A number of other studies have identified *T. forsythia* from dental root canals by polymerase chain reaction (25, 55, 107, 176, 195), DNA probes (59, 198, 213), or microarray (DNA chip) (249). It was detected in periapical tissues using fluorescent *in situ* hybridization (FISH) (**Fig. 5**) (212). Subgingival *T. forsythia* is the likely source in endodontic pathology (176), and for infections around dental implants (129, 130, 243); *T. forsythia* was associated with peri-implantitis by culture (223) and by DNA probes (98). The microbiota of failing implants was similar to that of refractory periodontitis (133).

Source of T. forsythia detection in children

An early indication that *T. forsythia* might be detected in children and transmitted from parent to child came from data using the BANA-based assay (183). *T. forsythia* was detected by polymerase chain reaction from tube-fed children (18), Down syndrome children (4), and

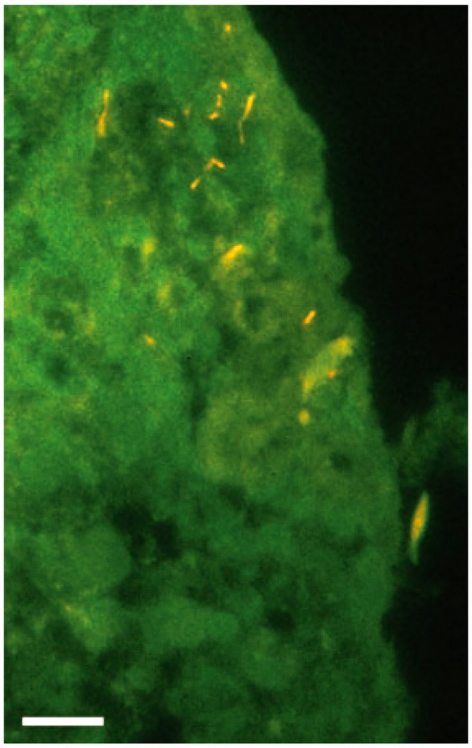

Fig. 5. Visualization of *Tannerella forsythia* by FISH on tissue sections from a periapical endodontic lesion. Simultaneous hybridization was performed with the bacteria-specific probe EUB 338-FTIC (green), and the species-specific probe *T. forsythia*-Cy3 (orange) (212). Reprinted with the permission of the Society for General Microbiology.

children with periodontitis (159), but was found infrequently in medically healthy children (118), also infrequently in children when using cultural detection (110, 111). It was detected using DNA probes in a population-based study of preschool children with a high prevalence of dental caries (227). In that population there were increased odds of species detection in the child if the child's parent was also positive (227). In the children, *T. forsythia* detection was associated with gingival bleeding, and a higher odds that *T. forsythia* would be detected if the mother had periodontitis (262). Polymerase chain reaction detection of *T. forsythia* in children was also linked to its detection in a parent (160, 218, 238). These studies suggested that *T. forsythia*, like other periodontal pathogens, is endogenous and primary infection likely occurs by vertical transmission.

While *T. forsythia* was detected in health, and gingivitis, the species was detected more frequently associated with disease than health, indicating, that in the oral cavity *T. forsythia* shows a defined association with periodontal, peri-implant, and endodontic pathology.

Cell and tissue invasion in humans

T. forsythia has been detected in host tissues; it was detected on or in crevicular epithelial cells from healthy and periodontally diseased patients (37) by DNA probes. The penetration of *T. forsythia* within the different layers of tissue has been shown by microscopy (177, 212). The progress made using FISH microscopy-based technology enables investigators to observe a fragment of tissue and selectively detect one or more organisms. Using a probe specific to one organism, or a probe that is universal to bacteria, the load and distribution of the organism within the sample can be observed. Such techniques have been applied successfully to periodontal tissues (174, 254), and to periapical lesions (Fig. 5) (212).

Localization of *T. forsythia* within buccal epithelial cells from subjects has been recently described using FISH (175). Surprisingly, the cells were neither monoinfected, nor were *T. forsythia*, *P. gingivalis* or *Actinobacillus actinomycetemcomitans*, the major invading bacteria. Other tissue-invading cells were species of *Streptococcus* (175). This finding suggests that a wide range of species may invade host cells, perhaps providing a strategy for bacterial persistence.

The increasing association of systemic disease with periodontal infection led to periodontal species being sought in extra-oral host tissues, mainly using polymerase chain reaction-based methods. Polymerase chain reaction was used to identify *T. forsythia* in atheromatous plaques (85), in coronary stenotic artery plaques (101), in atherosclerotic vessels (54), and in occluded arteries of patients with Buerger disease (103). *T. forsythia* was also detected in the bronchial tissues of embalmed cadavers (259). Future study will clarify whether this periodontal species is contributing to the pathology in these clinical conditions.

T. forsythia in animal models of infection

Mono- and mixed infections with *T. forsythia* in animal models provide an opportunity to examine the cooperative and competitive components of polymicrobial oral infections. Several animal models have been used to study the pathogenic potential of *T. forsythia* in periodontal and endodontic infections. As a mono-infection, *T. forsythia* was among the species, including *P. gingivalis* and *F. nucleatum*, that induced alveolar bone loss in gnotobiotic rat infections (220). Rabbits and mice were used to

test its pathogenicity in a subcutaneous abscess model (10, 216). More recently more technically challenging models of mouse gingival tissue (1, 7, 190), and endodontic infections have been used to mimic oral challenges and outcomes (8, 56, 185).

Mono- and mixed infections of *T. forsythia*, *P. gingivalis*, and *F. nucleatum* were tested in a wound chamber model in rabbits (216). While there was no abscess formation in mono-infections involving individual species, infection by *T. forsythia* plus *P. gingivalis* resulted in abscess formation in all tested animals, whereas *T. forsythia* and *F. nucleatum* mixed infections resulted in abscesses in 75% of the tested animals. The synergistic effect of *T. forsythia* and *P. gingivalis* was also observed in mice (265). These results indicated that the ability of *T. forsythia* to invade tissues is not only dependent on the characteristics of the strain (species) used but may result from interspecies interactions. A periodontal challenge of *P. gingivalis*, *B. fragilis*, *B. vulgatus*, *P. intermedia*, and *T. forsythia* resulted in alveolar bone loss and exposure of molar tooth roots (1). Using a mouse model for endodontic infection it has been shown that a mixed infection of *P. gingivalis*, *T. denticola*, and *T. forsythia* induced periapical bone loss (56).

From association with infection to the definition of a pathogen

Both clinical studies in humans, and animal model studies, associate *T. forsythia* with disease. Designating a bacterial species as a pathogen includes associating the species with disease and associating the species with pathogenicity-related factors that include host response, virulence factors, and molecular signatures (58, 170).

Determination of the cell components thought to be important in pathogenicity starts with identification of their effects on the host. Both the cell biology of *T. forsythia* and its effect on the host are still largely unknown. Three areas of research are underway:

- understanding the components of *T. forsythia* cell biology that are needed for a population to settle in a niche, which may also have deleterious effects on the host.
- identifying strategies of biofilm formation and maintenance.
- understanding the host–bacteria cross-talk that forms the basis of health, disease, and healing.

These three areas of research provide opportunities to intervene in disease-associated colonization and persistence. Not all of these components are considered traditional pathogenic factors, but they contribute to successful colonization and loss of biofilm control by the host. The availability of the genome sequence, a nascent genetic system, and animal models, provide tools to evaluate and define the pathogenic potential of a species, and create opportunities to experiment with new treatment strategies.

Genome sequence of T. forsythia

The full genome of *T. forsythia* type strain, ATCC 43037, has been sequenced. The strain was provided by F.E. Dewhirst and M.F.J. Maiden of The Forsyth Institute. The cloning, sequencing, and assembly of the genetic sequence were performed by Steven Gill and colleagues at the TIGR Institute. The primary annotation and web-site presentation are available through the Oral Pathogen Sequence Databases at Los Alamos National Laboratory Bioscience Division (http://www.oralgen.lanl.gov). The dynamic annotation compiling all available genetic sources is available through the Bioinformatics Resource for Oral Pathogens at The Forsyth Institute (http://www.brop.org) (19).

The genome contains 3,405,543 base pairs, and 3,034 predicted open reading frames. The GC content is 46.8%. Two 16S rRNA sequences are present in the genome. Phage and transposons are encoded within the genome but their function has not yet been investigated. Fifteen genomic (pathogenicity) islands were detected, including the *Bacteroides* conjugative transposon-related island, *tra* gene cluster (http://www.oralgen.lanl.gov). The *tra* genes are organized in the same order in *P. gingivalis* and *P. intermedia*. A detailed analysis of the genome sequence will be provided with the release of the primary publication.

Information from the genome sequence of a pathogen can expand the study of a specific species through new technologies and by comparing genetic potential with other organisms. Data from genome sequences facilitate the investigation of critical events in the cell biology, the infectious process, and dialog between host and pathogen, or between microorganisms. The major goal of genome-enabled infectious disease research is the development of novel diagnostics and therapeutic strategies, both requiring a deeper understanding of the genetic makeup of the organism and the host, and the organism's expression potential (57).

A genetic system in its infancy

Only one group so far has successfully created a *T. forsythia* strain with a genetically altered gene (92). Honma et al. (92) used a suicide plasmid and a triparental conjugation. The suicide plasmid was constructed in an *Escherichia coli* replicating plasmid that cannot replicate in *T. forsythia*, containing *bspA* (targeted gene for the genetic interruption) and *tet*(Q) for tetracycline antibiotic selection. Genetic exchange was enabled by *E. coli* RK231, supplying the conjugal transfer function, between the *E. coli* strain carrying the suicide plasmid and *T. forsythia* ATCC 43037. The resulting clone contained a *bspA* gene interrupted by the insertion of the full plasmid (92). New, more efficient methodologies for genetic engineering may arise from analyzing the genome sequence. No functional reporter gene has been published but it is suggested that *Bacteroides* reporter genes may be of use.

T. forsythia cell biology: the foundation for population survival and expansion

Integral components of pathogenicity frequently have their origin in bacterial cell physiology. Understanding the bacteria–host interactions that are important in pathogenicity start, for example, from knowing which proteases facilitate bacterial nutritional requirements while enhancing tissue destruction (178).

Molecular-method-based data complement information obtained from biochemical characterization. Functional characterization without gene identification, and vice versa, however, can provide only a weak understanding of the cell's potential and/or its activity in the environment where challenges lie. To understand the complexity of the host–pathogen relationships, we need to move beyond the study of isolated individual biological components (genes, proteins, etc.) to study how the individual components cooperate. This integrative knowledge involves a functional study of cell components, a genomewide understanding of cell functions, their relationships, and the effects of products and by-products on their environment. This may be related to the same organism (quorum sensing, attachment to human cell, methylglyoxal toxicity, etc.), another member of the biofilm (growth stimulation, bacteriotoxin production, genetic exchange mediating antibiotic resistance,

etc.), or the host (innate immune response stimulation, inflammation, bone resorption, etc.).

Several genes of *T. forsythia* were sequenced before the full genome was sequenced. These *T. forsythia* genes included: the sialidase *siaHI* (102), the superoxide dismutase *sodF* (97), an α-D-glucosidase (97), an *N*-acetyl-β-glucosaminidase (97) the outer membrane *RagA*-like gene (97), *groEL* (169), *prtH* protease with hemolysin activity (178), *bspA* (191), *susB* (97), a formate channel (A. Nagai and N. Itoh, GenBank deposit, unpublished data), EF-Tu (A. Nagai and N. Itoh, Genbank deposit, unpublished data), and an unusual sigma factor common to the members of the *Bacteroidetes* phylum (252).

Binding of pathogens to tissues can be mediated through fibronectin binding activity. Binding of *T. forsythia* cells to immobilized fibronectin and fibrinogen was decreased in fibronectin- and fibrinogen-coated microtiter plates in a *bspA* genetically engineered strain that did not express BspA (92). Purified recombinant BspA protein competes with binding of a *T. forsythia* wild-type strain to fibronectin and fibrinogen on fibronectin- and fibrinogen-coated microtiter plates (191). BspA is immunogenic in patients with periodontitis (191). The BspA protein belongs in the leucine-rich repeat (LRR) protein family. The amino acid sequence includes leucine-rich repeat motifs, which is a versatile protein-binding motif. Most proteins belonging to the LRR family interact via protein– protein interaction in a receptor–ligand context (108, 120). Methylglyoxal is a pathogenic mediator produced by *T. forsythia*; a by-product of metabolism, it has cytotoxic effects on bacteria and host (116, 144). It is principally associated with the glycolytic pathway and allows the cell to control the rate of carbon efflux (53). Methylglyoxal accumulates in cultures of a variety of microorganisms, and in human tissues and it reacts with proteins to form advanced glycation end products toxic for bacterium and host. In response, organisms frequently use multiple defensive metabolic mechanisms for detoxifying methylglyoxal (109). In the gingival crevicular fluid methylglyoxal was detected at higher concentrations in subjects with periodontal disease than in healthy subjects (116). The contribution of inflamed tissue to the total amount of methylglyoxal is unclear. This reactive compound may contribute significantly to tissue damage in periodontal disease.

The enzymatic activities of *T. forsythia* include a trypsin-like protease (71) source of the BANA test, a β-lactamase (246), as well as phos-

phatase, peptidase, sugar-degradation, and lipase used for identification (145, 179, 230, 232).

T. forsythia biofilm formation and maintenance

T. forsythia is a component of the biofilms found in the oral cavity. The dynamics of biofilm formation in oral infections is complex in both structure and composition over time. There are interactions between species, competition between species, in addition to host influences on biofilm organization. These interactions may differ during disease progression, persistence or remission. The spatial bacterial organization within dental plaque *in vivo* may result from co-aggregation-dependent growth stimulation between pairs of organisms in the biofilm (162). Co-adherence between species and species-tospecies communication may also be key components for biofilm organization, helping or establishing spatiotemporal development (48, 121). Understanding and elucidating the dynamics of biofilm composition and structure is an incremental process. Analysis of mono-species and two-species biofilms facilitates understanding the intricate communication and interrelationships of polymicrobial biofilms involved in oral health and diseases.

T. forsythia can form biofilms *in vitro* with *F. nucleatum* (190). The thickness and structure of *T. forsythia* biofilms is influenced by *F. nucleatum*. Both species co-aggregate when in a planktonic form (190), and this interspecies binding appears to be critical in the formation and structure of *T. forsythia– F. nucleatum* biofilms, and favors *T. forsythia* growth (190). This type of interaction was not observed for the biofilms formed by *T. denticola* with *P. gingivalis* (248) and by *Streptococcus oralis* with *Actinomyces naeslundii* (162). Could the *T. forsythia–F. nucleatum* interaction facilitate intergenic exchange? This question, of interest for all oral organisms, can be addressed by analysis of both available genomes followed by experimental exchange of DNA.

Both *F. nucleatum* and *T. denticola* can bind to *T. forsythia* (99). Co-aggregation between *T. forsythia* and *T. denticola* is influenced by two LRR proteins, BspA and LrrA, for *T. forsythia* and *T. denticola*, respectively (99). *In vitro* binding assay between the two proteins, BspA and LrrA, expressed and purified as recombinant full and truncated forms, shows that the N-terminal region of LrrA, but not the LRR domain, is involved in the binding process (99). Other surfaceexposed proteins may also be

involved in cell-to-cell attachment; *T. forsythia bspA* is regulated by contact when forming a biofilm with *F. nucleatum* (100). Decrease of *bspA* transcription compared with planktonic cultures occurs while *T. forsythia* is forming a biofilm by itself, or with *F. nucleatum* (100).

Host response to T. forsythia

Animal models and *in vitro* cell culture models reveal the aggressiveness of *T. forsythia* in infection, and host–bacteria interactions. The immune response to *T. forsythia* was examined using a mouse abscess model. Live and non-viable *T. forsythia* cells were injected subcutaneously in the dorsum on the mouse. The mice developed lesions consisting of granulomatous tissue with a large central core. There was primarily an innate immune response, neutrophils being the dominant leukocyte 1 day after injection (69). Consequently, there was no evasion of the innate host defense mechanism, unlike for *P. gingivalis* (69). The immune reaction was greater with larger skin lesions using live *T. forsythia* compared to non-viable organisms (69). Cell culture *in vitro* analysis of host–bacteria interactions allows investigation of mechanistic components of health, disease or the healing process. The inflammatory response of a macrophage/ epithelial cell co-culture to mono- and mixed infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* was recently investigated (11). The 1:5 ratio of macrophage: epithelial cells was chosen to represent an inflamed periodontium. In this model *P. gingivalis*, *T. denticola*, and *T. forsythia* stimulated the secretion of pro-inflammatory cytokines (interleukin- 1b, interleukin-6), chemokines (interleukin-8, RANTES), prostaglandin E^2, and matrix metalloproteinase- 9. These oral pathogens have strong potential for activating host-mediated destructive processes. No synergistic effect was observed for cytokine, chemokine, prostaglandin E_2, and matrix metalloproteinase-9 production between mixed and mono-infections (11).

Cell apoptosis induced by *T. forsythia* was first demonstrated using a whole cell extract (5), and later using a *T. forsythia* lipoprotein fraction obtained by Triton X-114 phase separation (87). The same lipoprotein fraction also induced dose-dependent IL-6 production by gingival fibroblasts and an epithelial cell line (87). The indirect effect of lipoproteins on disease progression has been hypothesized (87). Under this hypothesis, the membrane fraction containing lipoproteins of *T. forsythia* induced

pro-inflammatory cytokines from gingival fibroblasts, monocytes, and macrophages, resulting in periodontal tissue destruction, bone resorption, and release of enzymes that degraded the extracellular matrix. Accumulation of the membrane fraction of *T. forsythia* in inflamed areas could then kill epithelial cells and gingival fibroblasts through apoptosis. Other oral organisms use similar strategies, or produce factors that lead to cell apoptosis (5, 86).

An external serrated S-layer is easily distinguishable in stained cell cross-sections observed by electron microscopy (Fig. 3) (155, 177, 232). This S-layer structure is the first cell ultrastructure encountered by the environment and the host. S-layer proteins are immunogens in periodontitis patients (177). The S-layer also mediates hemagglutination, which is inhibited by *N*-acetylglucosamine, and heat denaturation of the proteins. Polyclonal antibodies directed towards the S-layer also inhibit hemagglutination, as well as cell adherence and cell penetration into KB-cell lines (cell lines derived from epidermal carcinoma of the mouth) (177). Pre-immunization of mice with S-layer protected these animals against abscess formation after challenge with live *T. forsythia* cells (177). The nature of the hemagglutinin is still unknown. At least one hemagglutinin was inhibited by *N*-acetylneuraminyllactose (158), and this/these proteins may differ from the 200/210-kDa protein described by Sabet et al. (177). Progress will be made by exploring relationships between gene, protein, and antigens to understand the organism's relationships with its environment.

The immune response to *T. forsythia* may have implications for another oral pathogen. Both *P. gingivalis* and *T. forsythia* were found to share common antigens providing some level of immune protection (247). This reactivity was observed when the primate *Macaca fascicularis* was vaccinated with killed *P. gingivalis*, or when New Zealand White rabbits were immunized with *P. gingivalis* or *T. forsythia*. This cross-reactivity, however, was not observed with *A. actinomycetemcomitans*. Antibodies to shared lipopolysaccharide epitopes among oral pathogens or members of the oral microbiota may account, in part, for the immune protection observed in immunized monkeys (247). A direct consequence of this crossreactivity could lead to modulation of the duration and intensity of infection. If the patient was already challenged by a particular pathogen, when another species infects that shares common epitopes, the progress of the population invasion and maintenance may

be significantly altered. This may account for some of the variation observed in host susceptibility to periodontal diseases. Additional population variation in periodontitis and caries susceptibility was attributed to genetic factors, based on studies in twins (13, 147).

Summary, perspective and future opportunities

In this review, we have described *T. forsythia* from the perspective of the microbiologist, and its associations with periodontal and other oral infections. To assess pathogenic potential one needs to go further than association with disease. One needs to associate pathogenic mechanisms and mediators with host response. The availability of the full genome sequence data for a strain of *T. forsythia* isolated from a progressing lesion of advanced periodontitis opens new horizons for our understanding of *Tannerella* sp.–*Tannerella* sp., *Tannerella* sp.–microbiome, and *Tannerella* sp.–host interactions. New technologies are becoming available to study this species and others in the genus, which will provide new insights into the cell biology of the bacteria and the host. The quantum leap provided by a full genome will greatly enhance the analytic power of proteomics, transposomics, genomics, and bioinformatics to be applied to this species. While the first steps will appear mainly to benefit basic science, translation researchers and clinicians will subsequently be able to incorporate those findings to improve care and disease prevention.

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