Uncultivated *Tannerella* BU045 and BU063 are slim segmented filamentous rods of high prevalence but low abundance in inflammatory disease-associated dental plaques

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Uncultivated clones BU045 and BU063 and *Tannerella forsythia*, a 'consensus periodontal pathogen', are the closest known relatives within the genus *Tannerella*. They have been described to inhabit different ecological niches of the human oral cavity. In this study, fluorescent *in situ* hybridization (FISH) and immunofluorescence were combined to investigate the prevalence and abundance of BU045 and BU063 in comparison to *T. forsythia* in plaques from gingivitis, necrotizing ulcerative gingivitis (NUG) and chronic periodontitis. Phylotype-specific FISH probes identified BU045 and BU063 as elongated thin rods with a segmented structure. Two structurally similar and previously unknown, rare phylotypes (127⁺ and 997⁺) were also identified due to partial 16S rRNA sequence identity with *T. forsythia*. In gingivitis, NUG and periodontitis patients, BU045, BU063, 127⁺, 997⁺ and *T. forsythia* were detected with prevalences of 50/83/71/14 and 81 %, 100/100/86/17 and 53 %, and 100/100/12/0 and 100 %, respectively.

Supragingivally, colonization density of all five organisms was generally low, rarely exceeding 0.1 % of the total biota. In periodontal pocket samples, however, cell numbers of *T. forsythia*, but not of the uncultivable phylotypes, were greatly elevated. Our data demonstrate that *Tannerella* phylotypes BU045, BU063, 127⁺ and 997⁺ consist of long slim rods with segments, which, with respect to FISH stainability, often behaved as independent units. The phylotypes are frequent but low-level colonizers of various periodontal disease-associated plaques. Their apparent inability to proliferate to high density seems to exclude any relevance for the pathogenesis of periodontal diseases.

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INTRODUCTION

Relying on comprehensive culture analyses, Moore & Moore (1994) estimated that, on a population basis, the human oral cavity might harbour some 500 bacterial species. With the emergence of culture-independent methods for the identification of microbial biota (see Amann *et al.*, 1995 for a review) it became clear that probably less than 50 % of the oral taxa have been cultured so far (Wilson *et al.*, 1997). During the last decade hundreds of previously unknown phylotypes and clones have been identified in studies investigating 16S rRNA diversity in different forms of dental plaque (Paster *et al.*, 2001, 2002; Munson *et al.*, 2004; Aas *et al.*, 2005; Kumar *et al.*, 2005; de Lillo *et al.*, 2006). Among the many uncultivable human oral phylotypes two clones with an apparently supragingival habitat, BU045 and BU063, attracted some interest, because their 16S rRNA gene

sequence groups them within the genus *Tannerella* (Paster *et al.*, 2001; Leys *et al.*, 2002; de Lillo *et al.*, 2004), which otherwise contains, besides some uncultivated clones from soil, only a single species, *Tannerella forsythia* (also known as *Tannerella forsythensis*) (Tanner *et al.*, 1986; Tanner & Izard, 2006). *T. forsythia* is a 'consensus periodontal pathogen' (Haffajee & Socransky, 2006) and associated with subgingival plaque from chronic and occasionally aggressive periodontitis (see Tanner & Izard, 2006, for review) rather than periodontal health like the two clones BU045 and BU063. Thus, although all three taxa colonize areas of the gums in the human oral cavity, current evidence indicates that they reside in quite distinct environments with markedly different nutrient sources.

The aims of the present study were to morphologically identify the uncultivable *Tannerella* phylotypes represented by clones BU045 and BU063, and to compare with *T. forsythia* their prevalence and abundance in plaques collected from patients with gingivitis, necrotizing ulcerative gingivitis (NUG) and chronic progressive periodontitis.

Abbreviations: FISH, fluorescent *in situ* hybridization; IF, indirect immunofluorescence; NUG, necrotizing ulcerative gingivitis.

Based on the available infrastructure, experience and DNA sequence information, we opted for a microscopic approach combining fluorescent *in situ* hybridization (FISH), using DNA probes to phylotype-specific 16S rRNA sequences, with indirect immunofluorescence (IF) to target the *Tannerella* organisms.

METHODS

Strains, dental plaque samples and cultivation. *T. forsythia* strains of oral bacteria were grown anaerobically (85 % N₂, 10 % H₂, 5 % CO₂) at 37 °C in fluid universal medium (FUM) (Gmür & Guggenheim, 1983) enriched with 5 % horse serum and 100 mg N-acetylmuramic acid l⁻¹ or in the chemically defined medium OMIZ-W68 with 100 mg N-acetylmuramic acid l⁻¹ (Wyss, 2007), the latter medium being superior in promoting growth of *T. forsythia*. Bacteria were harvested after 24–36 h from the exponential phase of growth.

Subgingival plaque samples (*n*=17) were obtained with paper points (Gmür *et al.*, 1989) from the deepest periodontal pockets (≥5 mm) of one or more quadrants of seven patients (five female) with a mean age of 59.1 years (range 50–71 years). The patients had been referred to our institute due to refractory chronic periodontitis (Armitage, 1999) for microbial testing at the sampled sites. Samples were processed for culture within 1 h from collection and for FISH and IF either within 1 h of collection or after storage at –80 °C and a single freezing/thawing cycle (Gmür & Thurnheer, 2002).

Pooled marginal supragingival plaque samples were derived from individual Chinese patients affected by gingivitis (*n*=7, four female) or NUG (*n*=7, four female) with a mean age of 35.9 years (range 27–53 years) and 32.6 years (range 27–52 years), respectively. They were taken from a previously described collection of frozen (liquid N₂) aliquoted samples (Gmür *et al.*, 2004) that had not been frozen/thawed more than twice before.

Preparation of multiwell slides for FISH and IF analyses. Suspensions of cultured bacteria (Gmür & Thurnheer, 2002) were washed in 0.9 % NaCl, diluted in coating buffer (0.9 % NaCl, 0.02 % NaN₃, 0.00025 % cetyltrimethylammonium bromide), spotted directly on 18- or 24-well slides (Cel-Line Associates) and air-dried. For FISH, slides were fixed by a 20 min incubation at 4 °C in 4 % paraformaldehyde/PBS; for IF, by a 2 min dip in methanol. Plaque samples were vortexed for 60 s at maximum speed and immediately diluted 1:8 in coating buffer. Thereafter, 10 µl plaque suspension per well was dropped onto 18- or 24-well slides, air-dried and fixed as described above. Slides, stored at room temperature, were processed for FISH or IF within 48 h from fixation.

FISH and IF procedures

FISH. Custom-synthesized oligonucleotide probes, labelled at the 5'-end with Cy3 or 6-FAM, were purchased from Microsynth. Probes were designed according to the criteria described by Manz (1999) using the ARB software (Ludwig *et al.*, 2004) (<http://www.arb-home.de>) and rRNA sequence information from the Ribosomal Data Base Project II (Cole *et al.*, 2005) (<http://rdp.cme.msu.edu/>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequences of the probes employed for the domain *Bacteria*, for *T. forsythia* and the *Tannerella* clones BU045 and BU063 are listed in Table 1. Probe EUB338 was used as universal positive control, but results are not further detailed below. Probes were used at final concentrations of 5 ng µl⁻¹ (Cy3 conjugates) and 15 ng µl⁻¹ (FAM conjugates) in the presence of 40 % formamide in the hybridization buffer. FISH was performed in 50 ml plastic centrifuge tubes at 46 °C as described by Thurnheer *et al.* (2001) except for the following modifications (Gmür & Lüthi-Schaller, 2007): (i) slides were not dehydrated in ethanol prior to hybridization, (ii) wells were covered for 60 min at 37 °C with Denhardt's solution (Sigma-Aldrich; diluted 1:50 in 0.9 % NaCl) in the presence of protectRNA RNase inhibitor (Sigma-Aldrich, diluted 1:500 in 0.9 % NaCl) prior to hybridization to prevent unspecific binding of the probes to the bacterial surface, (iii) reagent volumes for 4 mm wells were 3–5 µl, and (iv) hybridization was limited to 120 min.

Immunofluorescence. *T. forsythia* was stained with mAb 116BF1.2 (Werner-Felmayer *et al.*, 1988). mAb binding was visualized by consecutive incubations with biotinylated goat anti-mouse immunoglobulin G (Sigma) and streptavidin-FITC (Sigma), diluted 1:250 and 1:1500, respectively, in borate-buffered saline supplemented with 0.5 % skim-milk powder and 0.05 % Tween 20 (Werner-Felmayer *et al.*, 1988). Bacteria labelled by FISH or IF were enumerated by counting positive cells in 8–24 randomly selected viewing fields. If samples contained very low numbers of stained cells, half of a well (corresponding to 165 viewing fields) was screened. This counting procedure resulted in a lower detection limit of approximately 0.3 × 10⁴ ml⁻¹.

Combined IF and FISH. The combined application of the two assays was performed using a recently described experimental protocol (Gmür & Lüthi-Schaller, 2007). Cells were visualized with an Olympus BX60 epifluorescence microscope [Olympus Optical (Schweiz)] equipped with phase-contrast, an HBO 103 W/2 mercury photo optic lamp (Osram) and Olympus filter sets U-MNIBA (6-FAM, FITC), U-MA41007 (Cy3) and BX-DFC5 (6-FAM/FITC/Cy3). Colour micrographs were taken with a digital Olympus Camedia 3030 camera, transferred to an iMac G5 personal computer

Table 1. rRNA probe sequences, target sites and target taxa

Probe	Sequence (5'–3')	Site	Target	Source*
EUB338	GCTGCCTCCCGTAGGAGT	338–355	Most eubacteria	1,2
Tan1260a	CGCATCCGATCACTCGGT	1260–1278	<i>Tannerella</i> clone BU045	3
Tan1260b	TGCATCCGATCGCTCGGT	1260–1278	<i>Tannerella</i> clone BU063	3
Tfor127	CTCTGTTGCGGGCAGGTTAC	127–146	<i>T. forsythia</i>	3
Tfor439	CGTATCTCATTTTATTCCTGTGA	439–462	<i>T. forsythia</i>	4
Tfor582	GCGGACTTAACAGCCACCT	582–601	<i>T. forsythia</i>	5
Tfor997	TCACTCTCCGTCGTCTAC	997–1014	<i>T. forsythia</i>	3

*1, Amann *et al.* (1995); 2, Daims *et al.* (1999); 3, this study; 4, Sunde *et al.* (2003); 5, Gmür & Lüthi-Schaller (2007).

and processed using iPhoto 6.0.4 (Apple) and Photoshop 6.0 (Adobe) without qualitative changes to the raw images, except for contrast enhancement in Fig. 1(J).

Estimation of total bacterial cell numbers. The total cell number of samples from the Chinese gingivitis and NUG patients had been determined in a previous study by automated image analysis of fluorescence-labelled cells (Gmür *et al.*, 2004). Cell numbers present in periodontal pocket samples were determined by anaerobic cultivation for 5 days (37 °C) on Columbia blood agar (Oxoid) supplemented with 5% haemolysed human blood. In a previous study we have shown that numbers of fluorescent cells determined by visual microscopy and by image analysis correspond well, whereas total numbers of c.f.u. are on average about a factor of 3 lower than those determined by image analysis (Gmür *et al.*, 2000). Therefore, we multiplied the numbers of c.f.u. by 3 to generate the total cell number estimates used to calculate the phylotype proportions shown in Table 2 by a colour code.

Measurements of bacterial dimensions. The length of FISH-labelled *Tannerella* organisms was determined by assessing randomly selected cells on digital images with the imaging software cell^P 2.3 (Olympus). Images were made at 1000× magnification with an Olympus BX61 epifluorescence microscope equipped with phase contrast, an HBO 103 W/2 mercury photo optic lamp (Osram), filter sets 31001 (6-FAM, FITC), 41007a (Cy3) and 51004v2 (6-FAM/FITC/Cy3) (Chroma), and an F-View II Digital B/W camera (Olympus).

RESULTS

Morphology of BU045, BU063 and *T. forsythia*

Probes Tan1260a and Tan1260b, designed to detect *Tannerella* clones BU045 and BU063, respectively, were negative with all 13 *T. forsythia* strains tested. Applied to subgingival and supragingival plaque samples the two probes labelled exclusively elongated straight or irregularly bent thin rods with tapered ends. Marked cells were found both trapped in dense aggregates and separated from any other cells (Fig. 1A–C). The rods displayed a segmented structure. Quite frequently intensely stained segments were adjacent to terminal or inner segments without any FISH staining (Fig. 1E–H). Measurements with two samples from different periodontitis patients showed that the length of BU045 and BU063 cells varied over a broad range (Table 3). Moreover, sample P6 (26d) contained on average longer BU045 and BU063 cells than sample P5 (26d).

All 13 cultured *T. forsythia* strains were strongly positive with FISH probes Tfor127, Tfor439, Tfor582 and Tfor997 designed to detect *T. forsythia*, but not with the BU045- and BU063-specific probes Tan1260a or Tan1260b. In both subgingival and supragingival plaques, all four *T. forsythia* probes always stained slightly spindle-shaped rods (Fig. 1D, K and L) that were much shorter than BU045 and BU063 (Table 3) and frequently colonized with high density large multi-species aggregates (Fig. 1D). Double-labelling experiments with Cy3- and FAM-tagged probes showed, as expected, that the four probes for *T. forsythia* recognized the same cells (data not shown). Analogously, combined labelling by IF and FISH, using the *T. forsythia*-specific

mAb 116BF1.2 in combination with any of the six *Tannerella* probes, demonstrated that the antibody and the DNA probes Tfor127, Tfor439, Tfor582 and Tfor997 identified the same short spindle-shaped cells (see Fig. 1I and J for a representative pair of images). We never found any 116BF1.2-positive elongated cells.

Unexpectedly, probes Tfor127 and Tfor997 detected two further rare populations of very elongated segmented rods (Fig. 1M). These thin organisms were not stained by any other of the six *Tannerella* probes, suggesting that they belong neither to *T. forsythia sensu stricto*, nor to clones BU045 or BU063. They are designated in the following as phylotypes 127⁺ and 997⁺, whereby the numbers refer to the DNA probes used for their detection.

Prevalence and abundance in clinical samples of the detected micro-organisms

Table 2 describes with numerical data the prevalence and abundance of the so far uncultivable phylotypes BU045, BU063, 127⁺ and 997⁺, and of *T. forsythia* in samples from gingivitis, NUG and chronic periodontitis patients. In addition, a colour code indicates the percentages of these taxa with the estimated total cell number being set to 100%. The gingivitis and NUG samples contained on average total cell numbers of $2.6 \times 10^8 \text{ ml}^{-1}$ (range 4×10^7 – 7.4×10^8) and $7.6 \times 10^8 \text{ ml}^{-1}$ (range 2.9×10^8 – 1.4×10^9) whereas the periodontitis samples yielded on average $1.7 \times 10^7 \text{ ml}^{-1}$ (range 4.4×10^6 – 5.8×10^7) (data not shown). Read column-by-column, Table 2 shows that clone BU045 was identified by Tan1260a in gingivitis, NUG and periodontitis samples with a prevalence of 50, 83 and 81%, and clone BU063 (Tan1260b) with a prevalence of 100%, 100% and 53%, respectively. Both clones accounted in most samples for less than 0.1% of the total biota; in only two cases did BU063 exceed 1%. *T. forsythia* was detected with a 100% prevalence in the Chinese supragingival gingivitis and NUG plaques by three of the four rRNA probes and by the mAb. However, cell densities were consistently very low and often near the detection limit of these microscopic assays (approx. $0.3 \times 10^4 \text{ ml}^{-1}$). In the subgingival plaques from periodontitis patients, *T. forsythia* prevalence was 100% as well, but now all but three samples contained high levels of the organism. In more than half the samples *T. forsythia* exceeded 10% of the total biota. The IF assay consistently detected higher *T. forsythia* cell numbers than FISH. The two new phylotypes 127⁺ and 997⁺ never reached high densities. Phylotype 997⁺ was very rare, whereas 127⁺ occurred regularly in supragingival plaques, showing a prevalence of 71%, 86% and 12% in gingivitis, NUG and periodontitis samples, respectively.

DISCUSSION

This investigation has demonstrated that four uncultivated *Tannerella* phylotypes consist of slim, elongated, segmented

Fig. 1. Morphology of bacteria labelled in plaque samples by FISH or by both FISH and IF for *Tannerella* group organisms. (A and B) Phase-contrast and epifluorescence micrographs of a representative field of a periodontitis plaque stained with probes Tan1260a-Cy3 and Tan1260b-FAM. Cells of phylotypes BU045 (orange-red) and BU063 (green) are identified as elongated thin rods with tapered ends. (C) BU045 and BU065 cells in and around a large bacterial aggregate of NUG plaque (same staining as in B). We never observed bacteria of the two phylotypes to reach higher densities than in this field. (D) Aggregate from subgingival plaque stained with Tfor439-Cy3 for *T. forsythia*. Such focal high-density colonization by *T. forsythia* was common in subgingival plaques from severe chronic periodontitis. Note the difference in cell length in comparison to image C. (E and F) BU045 and BU063 cells marked by arrows have a segmented structure with some internal or terminal segments completely unlabelled by FISH. Same sample and same staining as in image B. (G and H) Magnification of the red-circled area of image E, illustrating the segmentation of a BU045 cell. (I, J) Area of a periodontitis plaque stained simultaneously for *T. forsythia* by IF (green) and FISH (orange-red) shows that mAb 116BF1.2 and probe Tfor582-Cy3 detect the same cells. (K, L) Representative field from a NUG sample labelled with Tfor439-Cy3, showing the characteristic morphotype of *T. forsythia*. (M) Another image of the same NUG sample but labelled with Tfor127-Cy3. Besides a cell with the standard morphology of *T. forsythia* (arrow), two much longer segmented cells are marked which otherwise are not labelled by any of the other probes and thus must belong to a further previously unknown *Tannerella* phylotype. Bars, 10 μ m.

rods with tapered ends. Among them are the two previously described clones BU045 and BU063 (Paster *et al.*, 2001; Leys *et al.*, 2002; de Lillo *et al.*, 2004) found in this study in supra- and subgingival plaques from patients with gingivitis, NUG or chronic periodontitis with prevalences between 50 and 100 %. The two other phylotypes are previously unknown taxa, which we tentatively designated 127⁺ and 997⁺ due to their sequence homology with *T. forsythia* in regions 127–146 and 997–1014, respectively, of the 16S rRNA gene. Their prevalence in periodontal pocket samples is very low, but at least 127⁺ has revealed high prevalence in the gingivitis and NUG samples. In contrast to *T. forsythia*, however, these uncultivable phylotypes never seem to proliferate to high densities and therefore are not likely to be important in the pathogenesis of these inflammatory periodontal diseases.

Inter-group differences in prevalence and abundance of the detected phylotypes should be interpreted cautiously since our study groups were small ($n=7$), and differed both in disease status and with respect to the patients' ethnic/geographical background. Nevertheless, it is noteworthy that our data on the prevalence and abundance of *T. forsythia* in the periodontal pocket microbiota are in agreement with earlier results from many different studies (see Tanner & Izard, 2006 for a review). With respect to supragingival plaques from gingivitis and NUG patients, the low abundance of *T. forsythia* had to be expected based on previous findings with supragingival plaque from different types of study groups (Gmür & Guggenheim, 1994; Paster *et al.*, 2002; Socransky & Haffajee, 2002). However, the observed 100 % prevalence in these samples is remarkable as it shows that *T. forsythia* is, even supragingivally, an ubiquitous organism in people affected by severe gingival inflammation. The critical question remains, however, what causes *T. forsythia* to proliferate to several orders of magnitude higher cell densities when they reach the environment of the periodontal pocket? It should be mentioned that our prevalence scores for supragingival plaques are higher than those reported by Socransky & Haffajee (2002) for both healthy subjects and patients with periodontitis. However, besides studying quite different patient groups, these authors used the checkerboard assay

with whole genomic probe hybridization, which may be less sensitive and has never been compared directly to our procedures. Reportedly, the checkerboard technique is optimized to detect bacteria in the range of 10^4 – 10^7 cells ml^{-1} (Socransky *et al.*, 2004), whereas the FISH and IF single-cell assays used in this study yielded data ranging between 2×10^3 and 3×10^8 (Table 2).

We noted that IF leads to higher *T. forsythia* cell counts than FISH when the cells are present in planktonic form, whereas the opposite is the case when cells were present in dense aggregates. The reasons for this are currently not fully understood; however, it seems likely that the small FISH probes penetrate aggregates consisting of packed cells and a diffusion-limiting extracellular matrix much more easily than the antibodies of the IF sandwich assay. Gradually impaired FISH-fluorescence of planktonic cells was presumably due to rRNA degradation during the preparatory steps of FISH, since we could greatly reduce, but still not totally control, the problem by adding an RNase inhibitor to FISH assay reagents. Certainly, the beneficial incorporation of an RNA-protective reagent into the FISH procedure will be of general interest to researchers investigating clinical and environmental microbial ecologies.

The unexpected finding that cells of the four uncultivated *Tannerella* phylotypes are thin segmented filamentous rods is interesting. Morphologically comparable cell types have been described as segmented filamentous bacteria (SFB) (Davis & Savage, 1974). SFB live in the small intestine of arthropods, fish, birds and many mammalian species including humans, where they are anchored to the epithelial surface (Tannock *et al.*, 1984; Smith, 1997; Meyerholz *et al.*, 2002). They are considered to be non-cultivable, species-specific, non-pathogenic, and potent activators of the mucosal immune system. However, based on the available 16S rRNA gene sequence data, the *Tannerella* phylotypes and the SFB belong to quite distinct phylogenetic groups (Snel *et al.*, 1995; Tanner & Izard, 2006) and labelling of any SFB by our FISH probes can be excluded. The length of cells from the uncultivable

Table 2. Grid plot describing the prevalence and abundance of FISH- or mAb-labelled *Tannerella* populations in plaques from three different patient groups: patients with gingivitis (G3–21), NUG (NUG33–49) or chronic progressive periodontitis (P1–7)

Tannerella phylotypes and *T. forsythia* were detected by six different FISH probes to specific 16S rRNA sequences and by antibody binding to a *T. forsythia*-specific surface antigen. Numbers in fields indicate counts ($\times 10^4$) ml^{-1} of positive cells; field colour indicates the percentage of positive cells with the determined total bacterial cell number set to 100 %. Key to the colour-code of fields: white with nt, not tested; white with –, no stained bacteria detected; grey, $\leq 0.1\%$ of all bacteria; green, between 0.1 and 1 % of all bacteria; yellow, 1–10 % of all bacteria; red, $> 10\%$ of all bacteria.

Disease group	Plaque sample	FISH with probe								IF with mAb
		Tan1260a	Tan1260b	Tfor127 long cells short cells		Tfor439 short cells	Tfor582 short cells	Tfor997 long cells short cells		
Gingivitis	G3	-	2*	-	-	nt	nt	-	-	26
	G10	nt	nt	0.4	4	0.2	nt	-	2	53
	G12	1.0	2	0.2	15	10	10	-	7	62
	G16	-	15	-	3	3	3	-	3	71
	G18	-	2	0.2	3	1.2	-	-	2	52
	G20	17	30	17	1.1	1.3	1.0	0.2	1.4	3
	G21	5	174	38	13	1.3	3	-	3	6
NUG	NUG33	4	87	12	14	4	3	-	4	47
	NUG35	23	37	33	49	15	9	13	94	150
	NUG37	0.8	15	6	6	5	5	-	5	210
	NUG38	-	26	2	0.4	1.2	-	-	0.8	8
	NUG39	0.2	53	0.8	0.4	0.4	-	-	2	60
	NUG43	0.8	106	0.6	8	8	8	-	5	130
	NUG49	nt	nt	-	0.4	-	-	nt	nt	26
Periodontitis	P1 (17m)	-	132	-	1320	1320	1320	nt	nt	1584
	P1 (48b)	0.7	0.2	-	358	80	325	nt	nt	1584
	P2 (17p)	1.0	0.1	0.3	3	2	1.1	-	0.7	339
	P2 (25m)	4	-	0.2	173	678	256	-	271	2931
	P2 (47d)	-	-	-	1.1	0.7	1.4	-	1.4	323
	P3 (17m)	0.2	2	-	133	135	109	-	142	276
	P3 (23d)	0.5	-	-	5	6	5	-	6	106
	P3 (47d)	1.3	6	-	36	38	27	-	33	162
	P4 (16d)	3	-	-	337	84	1089	-	99	1681
	P4 (37d)	-	-	-	1271	1320	630	-	1891	124
	P5 (26d)	23	2	-	2904	1985	2640	-	334	3476
	P6 (18m)	15	32	-	766	566	564	-	421	528
	P6 (26d)	57	47	-	244	255	384	-	276	348
	P6 (36d)	0.2	-	-	739	732	541	-	899	132
	P7 (11m)	-	-	-	440	468	484	-	420	660
	P7 (22d)	0.2	-	-	333	446	485	-	327	662
	P7 (36d)	2	1.1	-	710	718	535	-	779	1267
		Tannerella BU045	Tannerella BU063	New Tannerella phylotype	Tannerella forsythia	Tannerella forsythia	Tannerella forsythia	New Tannerella phylotype	Tannerella forsythia	Tannerella forsythia

Tannerella phylotypes was quite variable. It is our impression that the variable number of segments per cell could explain length variation; however, this hypothesis will need to be verified by electron microscopy. In contrast, *T. forsythia* cells were consistently short, 2.3 μm on average, with no evidence for segmentation. Occasionally, individual segments of apparently normal cells of BU045, BU063, 127⁺ and 997⁺ were not stainable by FISH. Estimated from such unstained segments, the length of individual segments is similar to the length of a single *T. forsythia* cell. The biological significance of such cell segmentation in the genus *Tannerella* remains to be elucidated. Lack of FISH staining of a single segment of a multi-segmented organism could reflect structural differences in cell wall composition,

resulting in greatly reduced probe permeability in an affected segment, or, perhaps more likely, indicate that such a segment contains no or much less rRNA than directly adjacent segments. This would imply that segments function and perhaps die as individual self-sustaining units. It would be interesting to test whether individual segments could give rise to new multi-segmented organisms; however, so far our attempts to cultivate BU045 and BU063 have not progressed beyond the stage of heterogeneous enrichment cultures (C. Wyss, personal communication).

Obtained with a new procedure developed for the purpose of this study (Gmür & Lüthi-Schaller, 2007), our combined FISH-IF experiments clearly demonstrated that the four

Table 3. Length of BU045, BU063 and *T. forsythia* bacteria in two subgingival periodontitis samples

Sample*	Bacteria stained by FISH with 16S rRNA probes†					
	Tan1260a	Tan1260b	Tfor127	Tfor439	Tfor582	Tfor997
P6 (26d)	8.0 ± 4.5 (32) 2.2–26.8	15.9 ± 7.0 (39) 4.1–28.9	2.4 ± 0.8 (53) 1.1–4.2	ND	ND	ND
P5 (26d)	4.9 ± 2.5 (33) 2.1–11.3	9.6 ± 4.4 (16) 3.4–20.5	2.2 ± 0.5 (170) 0.8–4.0	2.2 ± 0.5 (375) 1.0–4.7	2.3 ± 0.6 (261) 0.8–5.7	2.1 ± 0.5 (259) 1.0–4.1

*P6 and P5 are the patient codes. The epithet (26d) refers to the position of the sampled site in the dentition, i.e. the distal surface of the first molar in the second quadrant.

†The data are means ± SD and, in the line below, range of cell length in µm. The number of measured cells is given in parentheses. ND, Not determined.

FISH probes to *T. forsythia* and mAbs 116BF1.2 and 103BF1.1 (data for the latter not shown) all identify the same short rods with tapered ends. This again confirms the two antibodies' specificity, used in many studies since 1989 to enumerate *T. forsythia* in clinical samples (Gmür *et al.*, 1989, 2004; Gmür & Guggenheim, 1994; Kamma *et al.*, 2004). Notably, cells detected in plaque were usually shorter than cells derived from *in vitro*-grown *T. forsythia* strains (data not shown). The morphology of the identified *T. forsythia* cells is in agreement with earlier descriptions by Tanner *et al.* (1986) and Lai *et al.* (1987), but contrasts with the description by Gersdorf *et al.* (1993). These authors considered *T. forsythia* cells to be thin elongated rods, reminiscent of the uncultivable cells identified in this study. They used the 16S rRNA FISH probe BFV530 for detection, a probe that with the rRNA gene sequences available nowadays can be shown to hybridize to *T. forsythia* and to BU045 and BU063. Thus, most probably Gersdorf *et al.* (1993) had observed BU045 and/or BU063, and not *T. forsythia*, several years before the two phylotypes were (re)detected by partial sequencing of plaque-derived 16S rRNA clones (Paster *et al.*, 2001).

In conclusion, this study shows the value of combined targeted FISH and IF studies for the identification and quantification of elusive micro-organisms colonizing the human oral cavity. The study confirms the omnipresence of *T. forsythia* in plaques associated with different inflammatory periodontal diseases and its selective capacity to expand dramatically in the environment of the subgingival pocket. It further provides first evidence that still uncultivable human *Tannerella* phylotypes consist of elongated filamentous and segmented rods which colonize at low density both supra- and subgingival plaques of patients with severe inflammatory periodontal diseases.

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