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Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence *in situ* hybridization

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Background and Objective: Interactions between oral bacteria and gingival epithelial cells play an important role in the pathogenesis of periodontal diseases. This study used *in situ* hybridization with 16 rRNA probes and confocal microscopy to detect the periodontal pathogens *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *and Treponema denticola* within epithelial cells from periodontal pockets, gingival crevice, and buccal mucosa collected from subjects with chronic periodontitis ($n = 14$) and good periodontal health ($n = 8$).

Material and Methods: Each green fluorescent species-specific and universal probe was hybridized with all 58 epithelial samples from the 22 patients. The samples were observed by confocal microscopy to confirm the intracellular localization of oral species of bacteria. The mean frequency of detection and number of intracellular bacteria per epithelial cell were computed for each sample.

Results: The frequency of cells with internalized bacteria was higher in samples from the gingival crevice than in samples from the oral mucosa. Epithelial cells from all subjects harbored intracellular bacteria; however, patients with periodontitis presented significantly higher counts of bacteria per cell than periodontally healthy individuals ($p < 0.05$). Periodontal pathogens showed a trend to be detected in higher numbers in epithelial cells from periodontitis patients. In particular, *T. forsythia* and *T. denticola* were significantly more prevalent in periodontal pocket cells than healthy sulci and buccal cell samples in the periodontitis group ($p < 0.05$).

Conclusion: Those findings indicate that crevicular and buccal cells present internalized bacteria, regardless of periodontal status. However, higher bacterial loads are detected in cells from subjects with periodontitis.

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The persistent contact of subgingival biofilm bacteria with gingival crevice epithelial cells may play a crucial role in the initiation and progression from periodontal health to disease (1). In these processes, intracellular invasion of oral epithelial cells and gingival tissues is an important property of many oral species, including the periodontal pathogens *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Prevotella* spp. (2–10). Most of those studies, however, were performed *in vitro*, using few reference bacterial strains and either oral epithelial cell lines or primary epithelial cell or tissue cultures. Recently, Rudney and coworkers (11–13) employed fluorescence *in situ* hybridization–16S rRNA probes and laser-scanning confocal microscopy to identify intracellular oral species within human buccal epithelial cells *in vivo*. Nevertheless, very few investigations have determined bacterial invasion in crevicular epithelial cells obtained directly from periodontal sulci (14,15). In a previous study, we employed the Percoll density-gradient centrifugation and checkerboard DNA–DNA hybridization techniques to characterize the bacterial community associated with crevicular epithelial cells taken from sites with periodontitis and good periodontal health (15). A wide range of bacterial species was observed in or on those crevicular cells, but the methodology could not differentiate which species were attached to, or were within, the epithelial cells. Therefore, the present study used fluorescence *in situ* hybridization and laser-scanning confocal microscopy to detect the periodontal pathogens *P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans*, and *T. denticola* within epithelial cells from human gingival crevice and periodontal pocket and within buccal epithelial cells, taken directly from subjects with chronic periodontitis or with good periodontal health.

Material and methods

Human subjects

Fourteen subjects with chronic periodontitis were selected from a pool of patients who sought dental treatment at the Department of Periodontology of the Federal University of Rio de Janeiro. In addition, eight periodontally healthy volunteers participated in the study as controls. Periodontitis patients showed at least seven sites with probing pocket depth and clinical attachment level of ≥ 4 mm, whereas healthy controls showed no sites with a probing pocket depth of > 3 mm and clinical attachment level of > 4 mm. Exclusion criteria included pregnancy, systemic conditions that could affect the progression or treatment of periodontal diseases, and use of antibiotics 6 mo prior to entry into the study. All patients were informed about the nature of the study, and a signed consent form was obtained from each individual. The study protocol was approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital.

Clinical examination and sampling

Periodontal clinical measurements were performed at six sites per tooth at all teeth, excluding the third molars. The clinical measurements included probing pocket depth and clinical attachment level (mm), measured using a conventional manual periodontal probe (North Carolina probe; HU-Friedy, Chicago, IL, USA), and the presence or absence of supragingival biofilm accumulation and bleeding on probing. All clinical measurements were performed by one calibrated examiner. The sites to be sampled were isolated with cotton rolls and the supragingival biofilm was removed with a sterile gauze. A pooled epithelial cell sample was obtained from at least three sites with deep periodontal pockets, and a second pooled sample was obtained from three periodontally healthy sites in each periodontitis patient. The samples were collected by gently stroking the epithelial lining of the pocket or sulcus with a sterile curette. In the periodontally healthy subjects, a pooled sample of crevicular epithelial cells was obtained from three healthy sulci. In addition, buccal epithelial cells were collected with sterile cytological brushes from all 22 subjects. The epithelial cell samples were placed in sterile Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% bovine calf serum (Laborclin Ltda, Paraná, Brazil), L-glutamine (Gibco, Buffalo, NY, USA), L-cysteine (Sigma), gentamicin sulfate 50 μg/ml (Amersham Pharmacia Biothec do Brazil Ltda, São Paulo, Brazil), 2.0 μg/ml of amphotericin B (Bristol-Myers Squibb, New York, NY, USA) and processed immediately.

16S rRNA probes

The four species-specific oligonucleotide probes and the universal probe, EUB 338, which hybridizes with a conserved region of all eubacteria, were obtained as 5′ conjugates to the green fluorescent dye, fluorescein isothiocyanate (Bioneer Corporation, Rockville, MD, USA) (Table 1). The species *P. gingivalis* (ATCC 33277),

Table 1. Sequences of probes for fluorescence in situ hybridization of bacterial rRNA[a]

Probes	Sequences	References
Actinobacillus actinomycetemcomitans	5′-CAC CAG GGC TAA ACC CCA AT-3′	(16)
Porphyromonas gingivalis	5′-GGT TTT CAC CAT CAG TCA TCT ACA-3′	
Treponema denticola	5′-GCT CCT TTC CTC ATT TAC CTT TAT-3′	(17)
Tannerella forsythia	5′-TTC ACC GCG GAC TTA ACA-3′	(18)
EUB338 (universal for eubacteria)	5′-GCT GCC TCC CGT AGG AGT-3′	(19)

[a]References are relative to the *Escherichia coli* rRNA sequence.

T. forsythia (ATCC 43037), *A. actinomycetemcomitans* (ATCC 43718), and *T. denticola* (FDC B1) were used as controls for optimizing the hybridization assays with the species-specific and universal probes. *P. gingivalis*, *T. forsythia*, *and A. actinomycetemcomitans* were grown in blood agar supplemented with hemin (5 μg/ml), menadione (0.5 μg/ml) and *N*-acetylneuraminic acid (15 μg/ml) (Sigma), and *T. denticola* was grown in trypticase soy yeast extract-gelatin-volatic fatty acid-serum medium, and incubated at 37°C in an anaerobic chamber for 5 d. Each species-specific probe was tested against pure cultures of the respective target species (positive controls), as well as against unrelated bacterial species (negative controls), including *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 33780), *Staphylococcus aureus* (ATCC 3359), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella enterica* serovar Typhimurium (clinical isolate).

Fluorescence *in situ* hybridization and laser-scanning confocal microscopy

Each subject cell sample was hybridized separately with each species-specific and the universal probe. In addition, the strain *S. enterica* serovar Typhimurium (C-20, an invasive clinical isolate from the State University of Rio de Janeiro) was used as a positive control for adherence and invasion. *S. enterica* serovar Typhimurium was grown in brain heart infusion medium (Gibco) for 24 h at 37°C. Epithelial cell samples from one patient were infected with *Salmonella* in Dulbecco's modified Eagle's medium supplementaed with 10% bovine calf serum, L-glutamine, L-cysteine, gentamicine sulphate and amphotericin B, and incubated in 5% CO_2 at 37°C for 30 min, followed by hybridization and microscopic analysis. The fluorescence *in situ* hybridization protocol used was modified from that previously described by Rudney *et al.* (11). The epithelial cell samples and bacterial suspensions (positive and negative controls) were washed in sterile phosphate-buffered saline buffer and fixed in cold 100% acetone for 5 min. A 100-μl aliquot of fixed cells was spotted onto poly D-lysine-coated glass slides (Sigma) and permeabilized with 0.1% Triton X-100 (Sigma) for 10 min. A 100-μl aliquot of prewarmed hybridization buffer (0.02 M Tris-HCl, 0.9 M NaCl, 0.01% sodium dodecyl sulfate and 20% formamide, pH 7.6), containing 50 ng of each oligonucleotide probe, was carefully applied onto glass slides with epithelial cells or bacteria. Slides were incubated for 2 h in a dark humid chamber at 50°C. After hybridization, slides were washed twice for 15 min at 60°C with washing buffer (20 mM Tris, 180 mM NaCl and 0.01% sodium dodecyl sulfate). Slides were then rinsed with distilled water and air dried in the dark. Prolong Gold antifading reagent (Molecular Probes, Eugene, OR, USA) was added onto the glass slides, which were mounted and sealed with coverslips. Fluorescence from hybridized probes was visualized in an epifluorescence microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany), coupled to a Nikon Coolpix 4500 digital camera. Images were captured using a standard software package (Nikon Capture 4.4.0) provided with the digital equipment. A laser-scanning confocal microscope (LSM 510; Carl Zeiss) was employed in some samples to determine whether bacteria detected by fluorescence *in situ* hybridization were inside epithelial cells. A series of 24–28 confocal sections was scanned with increments of 0–1.5 μm, using an excitation wavelength between 488 and 533 nm. To determine colocalization of the universal and species-specific probes, *z*-section images at the red and green wavelengths were superimposed. Image processing and bidimensional and three-dimensional reconstructions were performed using the software ZEISS LSM IMAGE BROWSER, version 3.5.0.223 (Carl Zeiss). Images of z-stacks were captured in the green and red channels to minimize fluorescence crosstalk. This software was used to adjust the color balance and to optimize the visibility of bacteria with cells background and autofluorescence. All images were then saved as TIFF files.

Data analysis

All statistical tests were performed using the STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES (SPSS, Chicago, IL, USA), release 10.0. Full-mouth clinical measurements were computed for each subject and then averaged across subjects within the groups. Differences on clinical parameters between the two groups were sought using the Mann–Whitney, chi-square, and fluorescence *in situ* hybridization exact tests. Analysis of covariance (general linear model) was performed to investigate the possible effects of the covariates gender and age in the clinical parameters. The frequency of detection of each species was computed for each slide containing a cell sample type (periodontal pocket, healthy sulcus and buccal epithelial cells) of both periodontitis and control groups. At least four fields with epithelial cells (≈ 50–100 epithelial cells in total) were examined per slide. Thus, a sample was considered positive for a particular species when at least one epithelial cell in each field examined presented green fluorescent bacteria from the hybridized species-specific probe. Bacterial numbers were estimated by direct counting on fluorescence *in situ* hybridization images of the slides. The mean number of bacteria per epithelial cell was calculated for the four fields examined in each slide containing a cell sample, and then ranked in scores and recorded as follows: 0, no bacterial cells; 1, 1–20 bacterial cells; 2, 21–100 bacterial cells; 3, > 100 bacterial cells per epithelial cell. The frequency of the scores was computed for each cell sample within the groups. Significant differences in the prevalence of bacterial species associated with different epithelial cell types between the two groups were sought using the chi-square and Fisher's exact tests. Any difference of $p < 0.05$ was considered statistically significant.

Results

Fourteen adult patients with chronic periodontitis (mean age = 44.2 ± 1.4 yr; 57% men) and eight periodontally healthy individuals (mean age = 29.9 ± 2.3 yr; 21% men) participated in the study. The full-mouth clinical examination showed that patients with periodontitis presented a

significantly higher mean probing pocket depth of 2.8 ± 0.14 (mm), clinical attachment level of 3.5 ± 0.2 (mm), 47 ± 32% of sites with supragingival biofilm accumulation and 42 ± 29% of sites with bleeding on probing than periodontally healthy individuals (probing pocket depth, 1.6 ± 0.07 (mm); clinical attachment level, 1.6 ± 0.1 (mm); supragingival biofilm accumulation, 14.6 ± 9% of sites; bleeding on probing, 4 ± 4.3% of sites), even controlling for gender and age ($p < 0.01$, general linear model). The differences were more marked when the pooled sites selected for epithelial cell sampling from periodontitis (mean probing pocket depth, 5.8 ± 0.3; clinical attachment level, 6.8 ± 0.3; 86 ± 11% of sites with supragingival biofilm accumulation and 70 ± 18% of sites with bleeding on probing) and periodontally healthy subjects (mean probing pocket depth, 1.8 ± 0.1; clinical attachment level, 2.3 ± 0.1; sites with supragingival biofilm accumulation, 27 ± 15; and no sites with bleeding on probing) were compared ($p < 0.001$, Mann–Whitney *U*-test). A total of 175 and 68 slides containing epithelial cell samples from periodontitis and healthy subjects, respectively, were examined by the fluorescence *in situ* hybridization assay. Each of the species-specific probes hybridized with the respective target species, but not with heterologous species (negative controls) obtained from pure cultures. The universal probe hybridized with all bacterial cultures and epithelial cells invaded by the species *S. enterica* serovar Typhimurium (Fig. 1A,B). Table 2 shows the prevalence and numbers of bacterial cells detected in epithelial cells from periodontal pockets, crevicular sulci, and buccal epithelial cells of all 22 subjects. In general, the frequency of all four periodontopathogens was greater in samples from gingival crevice than oral mucosa. Moreover, most of the species were found to occur in the rank of 1–20 bacterial cells per epithelial cell. Subjects with chronic periodontitis presented significantly higher numbers of bacteria in all cell samples (> 100 bacterial cells) than periodontally healthy individuals, as demonstrated by the universal probe ($p < 0.05$; chi-square test). Likewise, the pathogens were detected in higher numbers (ranks 21–100 and > 100) in buccal epithelial cells, periodontal pocket and crevicular cell samples from periodontitis patients compared with healthy controls; however, the differences were not statistically significant. Regarding the frequency of detection, no significant differences between diseased and healthy subjects were observed for all cell samples (Table 2). Of interest, *T. forsythia* and *T. denticola* were significantly more prevalent in periodontal pocket cell samples than healthy sulci and buccal epithelial cell samples in the periodontitis group ($p < 0.05$; chi-square test). Fluorescence *in situ*

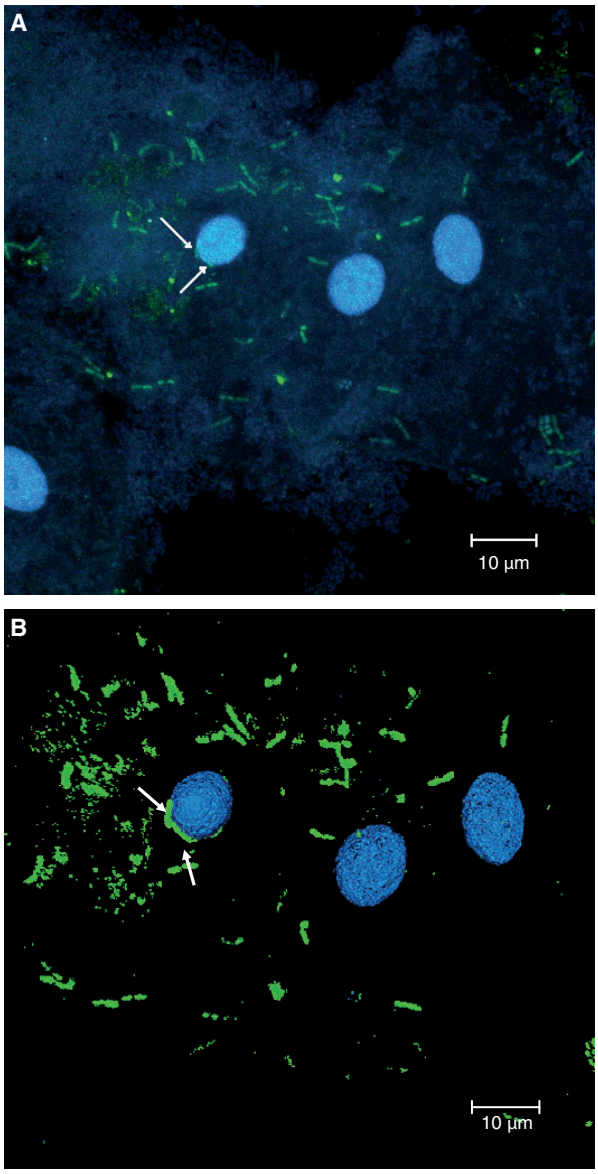


Fig. 1. Data from optimization experiments of fluorescence *in situ* hybridization (positive control for invasion). The panels show (A) fluorescence *in situ* hybridization of buccal epithelial cells from one patient presenting attached and internalized *Salmonella enterica* serovar Typhimurium. Brightly fluorescent cell-associated bacteria (arrows) are seen with the EUB338 probe (green). The blue channel was used for the cell background fluorescence. (B) Reconstructed Zeiss laser-scanning confocal microscopy image of the *z*-section (stack 92.2 µm; 7.2 µm) of (A) showing the intracellular *Salmonella* strain localized in the perinuclear region (arrow). Magnification, ×1000 (Objective Plan-fluar 100×/1.45).

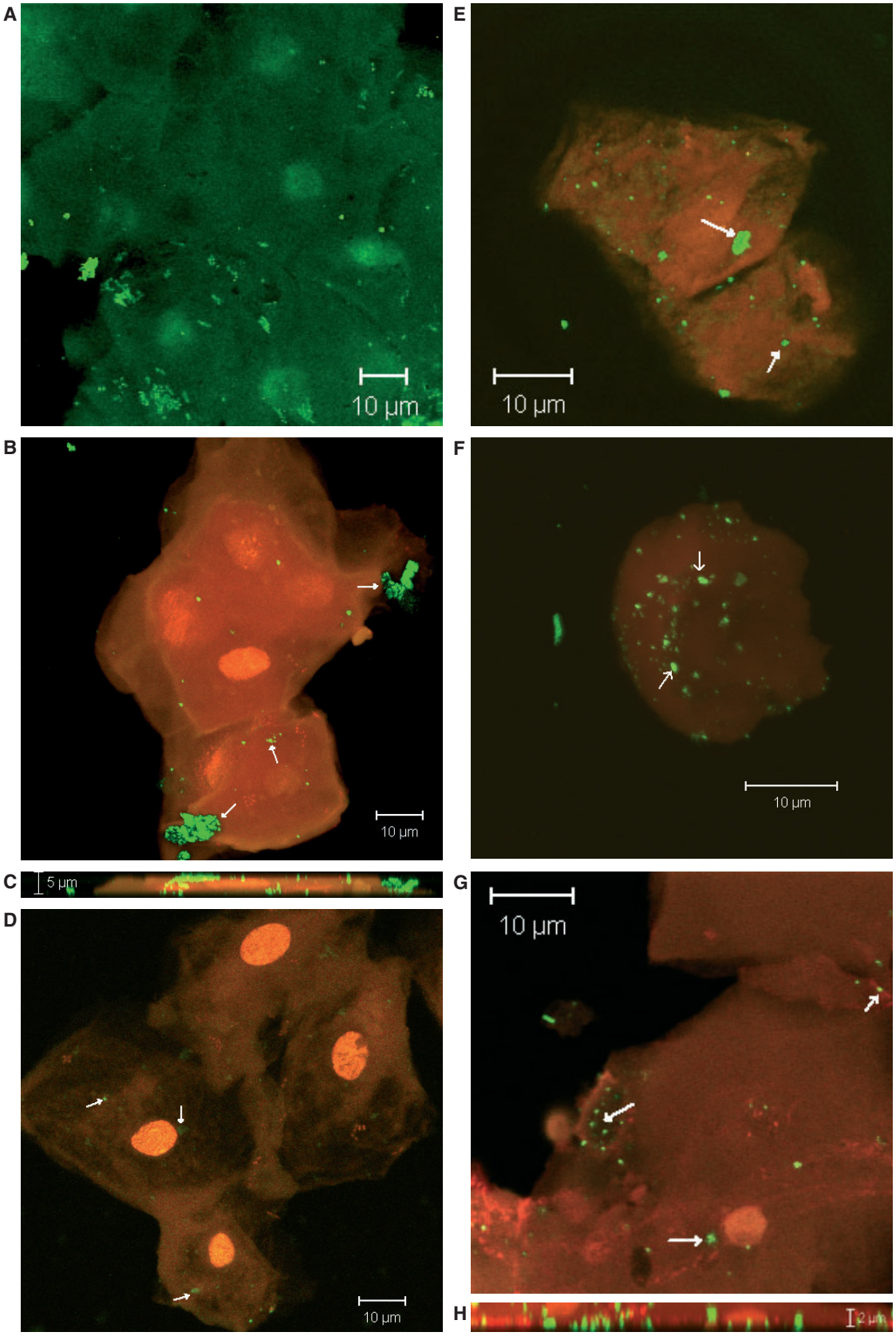
Table 2. Frequency of detection (%) and ranks of cell numbers of oral species detected by fluorescence *in situ* hybridization within epithelial cell samples from periodontal pockets, healthy sulci and buccal mucosa from chronic periodontitis and periodontally healthy subjects

	Chronic periodontitis subjects				Periodontally healthy subjects		
Oral species	DS[a] ($n = 14$)	HS[b] ($n = 14$)	BEC[c] ($n = 14$)	Total ($n = 42$)[d]	HS[b] ($n = 8$)	BEC[c] ($n = 8$)	Total ($n = 16$)[d]
Porphyromonas gingivalis	92.9	85.7	92.9	90.5	100	87.5	93.8
Rank[e]							
0	7.1	14.3	7.1	9.5	0	12.5	6.2
1–20	57.2	57.1	78.6	64.3	87.5	87.5	85.7
21–100	28.6	28.6	14.3	23.8	12.5	0	6.3
> 100	7.1	0	0	2.4	0	0	0
Tannerella forsythia[f]	92.9	64.3	42.8	66.6	62.5	75	68.7
Rank							
0	7.1	35.7	57.1	33.3	37.5	25	31.3
1–20	71.5	50	35.7	52.4	62.5	75	68.7
21–100	21.4	14.3	7.2	14.3	0	0	0
> 100	0	0	0	0	0	0	0
Treponema denticola[f]	92.9	78.6	42.8	71.4	75	62.5	68.7
Rank							
0	7.1	21.4	57.1	28.6	25	37.5	31.3
1–20	64.3	57.1	28.6	50	75	62.5	68.7
21–100	21.5	21.5	14.3	19	0	0	0
> 100	7.1	0	0	2.4	0	0	0
Actinobacillus actinomycetemcomitans	92.9	85.7	71.4	83.3	75	62.5	68.7
Rank							
0	7.1	14.3	28.6	16.6	25	37.5	31.3
1–20	64.3	57.1	42.8	54.8	62.5	62.5	62.5
21–100	28.6	28.6	28.6	28.6	12.5	0	6.2
> 100	0	0	0	0	0	0	0
Universal probe EUB 338	100	100	100	100	100	100	100
Rank							
0	0	0	0	0	0	0	0
1–20	7.1	7.1	7.1	7	12.5	12.5	12.5
21–100	57.1	57.1	71.5	62	87.5	75	81.3
> 100[g]	35.8	35.8	21.4	31	0	12.5	6.2

[a]DS, epithelial cell samples from sites with a periodontal pocket.
[b]HS, sites with periodontal health.
[c]BEC, buccal epithelial cells.
[d]Total number of epithelial samples per subject group.
[e]Ranks are presented as number of bacteria per epithelial cell.
[f]$p < 0.05$; refers to significant difference in frequency among cell samples within the periodontitis group (chi-square test).
[g]$p < 0.05$; refers to significant difference in the frequency of numbers of bacteria (> 100) between periodontitis and healthy subjects (chi-square test).

hybridization images by fluorescence microscopy cannot distinguish intracellular bacteria from bacteria on cell surfaces. Thus, buccal epithelial cell samples from a patient infected with an invasive *Salmonella* strain were hybridized with the universal probe and showed bright green fluorescence (Fig. 1A). Intracellular localization was confirmed by confocal microscopy, as depicted in the reconstructed image of the *z*-section of Fig. 1A, showing > 100 internalized bacterial cells localized in the perinuclear region (Fig. 1B). In addition, 32 cell samples from periodontitis patients and 10 samples from controls were selected for confocal analysis. Images of laser-

Fig. 2. Images of *z*-axis sections of epithelial cell samples from subjects with periodontal health and periodontitis. These examples are representative of the most common scoring pattern observed in this population. (A) Green fluorescent intracellular bacteria (> 100 bacteria) can be seen with the EUB338 probe. (B) Clusters of green fluorescent *Porphyromonas gingivalis* (arrows) were detected, using the species-specific probe, within epithelial cells from a periodontal pocket. The red channel was used for the epithelial cell background. (D) *Actinobacillus actinomycetemcomitans* (arrows) detected within cells from a healthy site and (E) from a periodontal pocket. (F) *Treponema denticola and* (G) *Tannerella forsythia* were detected with species-specific probes (green fluorescence) within epithelial cells from periodontal pockets. *z*-plane slices of three-dimensional reconstructions were generated to confirm intracellular bacteria and indicate clusters of intracellular *P. gingivalis* (C) and *T. forsythia* (H). Magnification, ×1000.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scanning confocal microscopy, presenting examples of epithelial cell samples from diseased and healthy subjects hybridized with the four species-specific probes and the universal probe, are depicted in Fig. 2A–H. These samples represented the most common scoring pattern (1–20 bacterial cells per epithelial cell) observed for these species (Fig. 2A). The universal probe showed that every cell sample tested harbored internalized bacteria when visualized by confocal microscopy (data not shown). In addition, the intracellular periodontal species appeared free in the cytoplasm or aggregated in clusters (Fig. 2B–2H). Epithelial cells from periodontal pockets (Fig. 2E) tended to present higher numbers of intracellular periodontopathogens, such as *A. actinomycetemcomitans*, than cells from healthy sites (Fig. 2D). *z*-plane slices of the image sections indicate the intracellular location of *P. gingivalis* (Fig. 2C) and *T. forsythia* (Fig. 2H).

Discussion

A great deal of evidence has indicated that intracellular invasion by oral bacteria has an important role in the etiology and pathogenesis of periodontitis (1). The proximity of bacterial products to host tissues, and the invasive mechanisms, lead to periodontal tissue destruction. In addition, internalization shields bacteria from the host immune system, providing an ideal environment for their survival and potential spread to other sites of the body (2,5,10,20). The question therefore arises of whether internalization is a common mechanism for oral species in both periodontal disease and health. Within this context, the present study determined the prevalence and numbers of four putative periodontal pathogens within human epithelial cells taken directly from periodontal lesions, gingival crevice and buccal mucosa of patients with periodontitis or periodontal health. The fluorescence *in situ* hybridization−16S rRNA probes and laser-scanning confocal microscopy methods were employed for visualization, identification, enumeration, and localization of the bacterial species within the epithelial cells. These techniques have been shown to be powerful tools for studying complex microbial communities and their relationship with different environments and hosts (21). A major advantage of these methods is the possibility of detecting growing bacteria, particularly unculturable or hard-to-grow species, such as several periodontal species. Overall, intracellular bacteria were detected in all epithelial cell samples. Similar results were demonstrated by other studies, suggesting that several oral species, including unidentified species, have the ability to invade (11–13). In fact, there is strong evidence to indicate that oral epithelial cells are capable of supporting surprisingly large bacterial populations (11,12). Although the prevalence of epithelial cells with internalized bacteria was similar in samples from both individuals with periodontitis and periodontally healthy subjects, the numbers of bacteria per cell were significantly higher in samples from individuals with periodontitis than in samples from healthy subjects. Conceivably, subjects with periodontal disease are more readily infected with a wide range of bacteria owing to the presence of multiple sites harboring high bacterial loads, including periodontal pockets, supragingival biofilm and oral mucosa (22–24). These sites provide a constant source for recolonization and cross-infection within the mouth, which may result in persistence of periodontal infections and/or dissemination of bacteria to other host sites (25). In fact, *P. gingivalis* has been shown within endothelial cells and atherosclerotic plaque, supporting the evidence of association between periodontal disease and systemic infections (3,26). Regarding the specificity of the intracellular microbiota, no significant differences in the prevalence and numbers of internalized periodontopathogens were found between the subject groups. However, these species showed a trend to be detected at higher frequency and numbers in periodontal pocket cells than in cells from healthy subjects. Likewise, crevicular epithelial cell samples seemed to harbor these microorganisms in higher numbers than buccal epithelial cells. Lack of significance may have occurred as a result of the small size of the sample population, as well as difficulties in counting internalized bacteria presented in clusters. Both *A. actinomycetemcomitans* and *P. gingivalis* were commonly found within epithelial cells from all subjects, supporting the invasive properties of these pathogens (4,6–8,10,11). In contrast, intracellular *T. denticola* and *T. forsythia* were observed in lower numbers, mainly in cells from patients with periodontal health. Other investigations reported that *T. forsythia* showed a weak ability to invade cells in tissue culture (5), but were not the dominant bacteria in buccal cells (12). Also, *T. denticola* has not been detected within buccal cells (13) and can pass through layers of cultured epithelial cells without entering cells directly (27). We found these species to be significantly more prevalent in periodontal pocket samples than in samples from healthy sulci and buccal epithelial cells in patients with periodontitis, confirming our previous data (15).

The findings presented in this investigation indicate that the intracellular location of a broad range of oral bacteria in different types of oral epithelial cells is a common phenomenon, regardless of periodontal status. However, cells from sites with periodontitis harbor higher numbers of internalized bacteria than other oral epithelial cells. Furthermore, some pathogens tended to occur in higher numbers within cells from periodontal pockets. One can speculate that, in the periodontal pocket site, the persistence of an inflammatory process associated with the establishment of a more pathogenic subgingival microbiota may induce epithelial cells to express specific molecules that favor the adherence and internalization of a greater number of certain bacterial species (20). Thus, changes in the composition and levels of the intracellular microbiota associated with gingival crevice cells may lead to a shift from a host-compatible to a disease condition. Finally, additional studies of the mechanisms involved in the complex interactions between a wider range of subgingival species and gingival crevicular cells in

periodontal health and disease are required for determining the relevance of bacterial invasion in periodontal infections.

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