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Association of *Eubacterium nodatum* and *Treponema denticola* with human periodontitis lesions

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Background: The purpose of the present investigation was to compare the levels, proportions and percentage of sites colonized by 40 bacterial species in subgingival plaque samples from periodontally healthy subjects and patients with chronic periodontitis to seek possible pathogens other than the consensus pathogens *Porphyromonas gingivalis* and *Tannerella forsythia*.
Method: Subgingival plaque samples were taken from the mesial aspect of each tooth in 635 subjects with chronic periodontitis and 189 periodontally healthy subjects. The samples were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization (total samples = 21,832). Mean counts, % DNA probe counts and percentage of sites colonized at $>10^5$ were determined for each species in each subject and then averaged in each clinical group. Significance of difference between groups was determined using the Mann–Whitney test. Association between combinations of species and periodontal status was examined by stepwise logistic regression analysis. Analyses were repeated using a subset of subjects from both clinical groups who had proportions of *P. gingivalis* plus *T. forsythia* less than the median (4.42%) found in periodontally healthy subjects. All analyses were adjusted for multiple comparisons.
Results: For the 824 subjects the consensus pathogens *P. gingivalis* and *T. forsythia* as well as *Eubacterium nodatum* and *Treponema denticola* had significantly higher mean counts, proportions and percentage of sites colonized in samples from subjects with periodontitis than from periodontally healthy subjects. There were significantly more *Capnocytophaga gingivalis*, *Streptococcus gordonii* and *Veillonella parvula* in periodontally healthy subjects. *E. nodatum*, *T. denticola*, *Streptococcus oralis*, *Streptococcus intermedius*, *Fusobacterium nucleatum* ssp. *vincentii* all had higher counts and proportions in diseased than healthy subjects who had low proportions of *P. gingivalis* and *T. forsythia.* Logistic regression analysis indicated that the same species groups were associated with disease status after adjusting for the proportions of the other species.
Conclusions: This investigation confirmed the strong association of *P. gingivalis* and *T. forsythia* with chronic periodontitis and emphasized a strong association of *E. nodatum* and *T. denticola* with periodontitis whether in the presence or absence of high levels of the consensus pathogens. Other species, including *S. oralis*, *Eikenella corrodens*, *S. intermedius* and *F. nucleatum* ssp. *vincentii*, were associated with disease when *P. gingivalis* and *T. forsythia* were present in low proportions.

Key words: periodontitis; periodontal health; periodontal pathogens; *Treponema denticola*; *Eubacterium nodatum*; subgingival microbiota

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In 1996, the World Workshop in Periodontics defined three bacterial species as etiological agents of periodontal diseases (19). *Actinobacillus actinomycetemcomitans* was considered to be a pathogen of various forms of aggressive periodontitis, while *Porphyromonas gingivalis* and *Tannerella forsythia* were considered to be important etiological agents of chronic periodontitis. These conclusions were based on a 'weight of evidence' evaluation which included factors such as association of the species with disease, the effect of elimination/suppression of the species on disease progression, the production of virulence factors that were plausibly related to disease pathogenesis, the production of an antibody response to the species during periodontal infection, the effect of the species in experimental animal model systems, and the risk of disease progression in subjects or sites harboring species at elevated levels (40, 106, 107).

Since 1996, the literature regarding the microbiota of periodontal diseases has emphasized these three pathogens. A search of *PubMed* reveals that since 1996, 65% of all papers published on the microbial etiology of periodontal diseases involved at least one of these three species. These papers have strengthened the link between these microorganisms and periodontal infections through new studies of the association between the presence and levels of these bacteria and signs of periodontal disease (25, 43, 47, 51, 60, 85, 90, 101), improvements in periodontal status associated with the concomitant reduction in prevalence and levels of these pathogens (20, 23, 53, 83), detection of new virulence factors for the three species (1, 3, 6, 8, 26, 28, 32, 33, 44, 76, 88), and studies of risk assessment (11, 30, 42, 56, 87).

Despite the intense focus on the 'usual suspects', some microbiological studies of the last decade have provided additional evidence of the etiological role of other putative pathogens and have suggested a few new candidates. The vast majority were studies of association that confirmed the relationship between species already considered possible pathogens and the etiology of periodontal diseases, including: *Prevotella intermedia*, *Prevotella melaninogenica*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Eikenella corrodens*, *Prevotella nigrescens*, *Capnocytophaga gingivalis*, *Treponema denticola*, *Treponema socranskii*, *Veillonella parvula* and *Campylobacter rectus* (16, 22, 31, 35, 54, 66, 67, 73, 89, 90, 98). Other species have been less commonly reported to be associated with periodontal diseases, including: *Eubacterium saphenum* and *Mogibacterium timidum* (59); *Prevotella corporis*, *Prevotella disiens* and *Peptostreptococcus magnus* (72); *Eubacterium nodatum* and *Slackia exigua* (10); and *Enterococcus faecalis*, *Escherichia coli* and *Bartonella sp*. (18).

More recent studies have attempted to seek or discriminate additional periodontal pathogens. Kumar et al. (49), using polymerase chain reaction (PCR), observed associations with chronic periodontitis for several new species or phylotypes, including uncultivated clones from the following phyla: *Deferribacteres*, *Bacteroides*, OP11 and TM7, as well as the named species *E. saphenum*, *Porphyromonas endodontalis*, *Prevotella denticola* and *Cryptobacterium curtum* (49). Using PCR of the 16S rRNA region, Dewhirst et al. (24) found a highly assorted *Treponema* population in the periodontal pockets of subjects with periodontitis, consisting of both cultivated and uncultivated species including *T. denticola*, *Treponema maltophilum* and *Treponema* sp. *Smibert-3*. Wyss et al. (93–97) isolated five novel species of treponemes from periodontal lesion samples and proposed the following nomenclature for the newly discovered spirochetes: *Treponema amylovorumin* sp. nov., *Treponema parvum* sp. nov., *Treponema lecithinolyticum* sp. nov., *Treponema putidum* sp. nov. and *Treponema maltophilum* sp. nov. A molecular epidemiological analysis using a *T. lecithinolyticum*-specific probe showed this organism to be associated with diseased periodontal sites when compared with non-diseased sites of periodontitis patients (95). A potential role of *Exiguobacterium aurantiacum* in periodontal disease has also been suggested (107).

Thus, the literature since 1996 confirmed the etiological role of the designated pathogens, *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* and extended the list of possible periodontal pathogens. However, many of the studies proposing new periodontal pathogens were association studies that examined small numbers of subjects, samples and/or species. The present investigation attempted to seek additional periodontal pathogens by examining the subgingival microbiota in large numbers of subjects who were periodontally healthy or exhibited chronic periodontitis. In examining the microbiological data from the subjects with chronic periodontitis, it became apparent that many of these subjects had relatively low levels and proportions of the consensus pathogens, *P. gingivalis* and *T. forsythia*. Indeed, many of the subjects had levels of these species that were as low as, or lower than, subjects who were periodontally healthy. It is possible that *P. gingivalis* and/or *T. forsythia* might have been pathogens in the subjects with low levels of these species, but that the species were either unusually virulent or the host was unusually susceptible to the organisms. Alternatively, and perhaps more likely, there were other species that were causally associated with disease in these individuals. The purpose of the present investigation was to examine subgingival plaque samples in chronic periodontitis and in periodontally healthy subjects to detect species other than *P. gingivalis* and *T. forsythia* that are associated with chronic periodontitis.

Material and methods

Subject population

A total of 824 adult subjects, 635 with chronic periodontitis and 189 who were periodontally healthy, were included in this retrospective analysis. All subjects had been enrolled in clinical studies at The Forsyth Institute in Boston or the University of Göteborg, Sweden and had signed informed consent before entry into their respective studies. Subjects ranged in age from 24 to 82 years of age and had at least 14 natural teeth. Subjects with chronic periodontitis exhibited at least four pockets of ≥4 mm and at least four sites with attachment level measurements of at least 3 mm or more. Periodontally healthy subjects exhibited no pocket depth or attachment level measurements >3 mm and had <30% sites exhibiting bleeding on probing. Exclusion criteria included pregnancy, nursing, periodontal therapy and antibiotic administration within the previous 3 months, as well as any systemic condition which might have affected the progression of periodontitis. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. No subjects with localized aggressive periodontitis, rapidly progressive periodontitis or acute necrotizing ulcerative gingivitis were included in the study. Demographic parameters as well as smoking histories were obtained using a questionnaire. Members of the study teams reviewed all answers with the subject.

Clinical measurements

Measures of plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (0/1), suppuration (0/1), probing pocket

depth and probing attachment level were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding the third molars at a baseline visit. Pocket depth and attachment level measurements were made using a North Carolina probe. The pocket depth and attachment level measurements were repeated and the means of the pairs of measurements were used in the analyses. The baseline clinical parameters for the chronic periodontitis and periodontally healthy subjects are presented in Table 1.

Microbiological assessment

Subgingival plaque samples were taken at baseline from the mesiobuccal aspect of all teeth (excluding third molars) in all subjects. Samples were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. Counts of 40 subgingival species were determined in each plaque sample using a modification (37) of the checkerboard DNA–DNA hybridization technique (81). In brief, after the removal of supragingival plaque, subgingival plaque samples were taken using individual sterile Gracey curettes from the mesial aspect of each tooth. The samples were placed in separate Eppendorf tubes containing 0.15 ml TE (10 mmol/l Tris–HCl, 1 mM EDTA, pH 7.6) and 0.15 ml of 0.5 M NaOH was added. The samples were lysed and the DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes for 40 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using alkaline phosphatase-conjugated antibody to digoxigenin and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL) and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe–target hybridization. Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm Fluorimager and converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero. A total of 21,832 subgingival samples were evaluated.

Data analysis

Microbiological data available for each subject were the counts of 40 test species in subgingival biofilm samples taken from the mesial aspect of up to 28 teeth. The counts for each species were averaged within each subject and then averaged across subjects in the two clinical groups. In a similar fashion, the percentage of the total DNA probe count was determined for each species at each site in each subject and averaged within each subject and then across subjects in the two clinical groups. Prevalence was computed by determining the proportion of sites in each subject colonized by each species at counts $>10^5$ and then averaging these proportions across subjects in the two groups. Significance of difference between groups was determined using the Mann–Whitney test and adjusted for multiple comparisons (79). For some plots, the mean values for each species in the two clinical groups were depicted as the mean ± 95% confidence intervals adjusted for 40 comparisons.

The proportion of periodontally healthy and chronic periodontitis subjects for whom the mean percentage of the total DNA probe count was in the upper quartile for each species distribution was determined by computing the upper quartile for each species and then determining the percentage of subjects in each clinical group that exceeded this value for each species. Significance of differences in percentage of subjects exhibiting elevated proportions of each test species was determined using chi square analysis and adjusted for multiple comparisons.

Percentile plots of the mean percentage of the total DNA probe count of selected species or combination of species were prepared as described by Cleveland (17). The significance of differences between distributions found for periodontally healthy subjects or subjects with periodontitis was determined using the Kolmogorov–Smirnov test.

Logistic regression analysis was used to evaluate associations between the mean percentage of the total DNA probe counts of each species and periodontal disease status (health/periodontitis). The analysis examined only main effects with forward stepwise selection of variables. Variable selection terminated when the variable entering the model had a P value >0.05.

Table 1. Mean (±SD) clinical parameters of periodontally healthy subjects and subjects with chronic periodontitis

	Health	Periodontitis	Mann–Whitney *P*-value
N	189	635	
Age (years)	37 ± 10	50 ± 11	<0.001
Number of missing teeth	0.8 ± 1.5	3.1 ± 3.0	<0.001
% Males	37	54	<0.001
% Sites with			
Plaque	48 ± 52	75 ± 51	<0.001
Gingival redness	31 ± 23	69 ± 35	<0.001
Bleeding on probing	18 ± 16	42 ± 26	<0.001
Suppuration	0 ± 0	2 ± 5	<0.001
Mean PD (mm)	2.26 ± 0.32	3.42 ± 0.81	<0.001
Mean AL (mm)	2.00 ± 0.65	3.53 ± 1.14	<0.001
% Current smokers	13	28	<0.001

PD, pocket depth; AL, attachment level.

Results

Comparison of the subgingival microbiota in health and disease for all subjects

Figure 1 presents the mean counts ($\times 10^5$), the mean percentage of the total DNA probe count and the mean percentage of sites with counts $>10^5$ of the 40 test species in subgingival plaque samples from the 189 periodontally healthy and 635 chronic periodontitis subjects. Species that differed at $P < 0.001$ after adjusting for multiple comparisons using all three data presentations included the members of the red complex, *T. forsythia*, *P. gingivalis* and *T. denticola* as well as *E. nodatum*. The ratio of the mean counts, proportions and percentage of sites colonized by the tested species in health and disease are presented in Fig. 2.

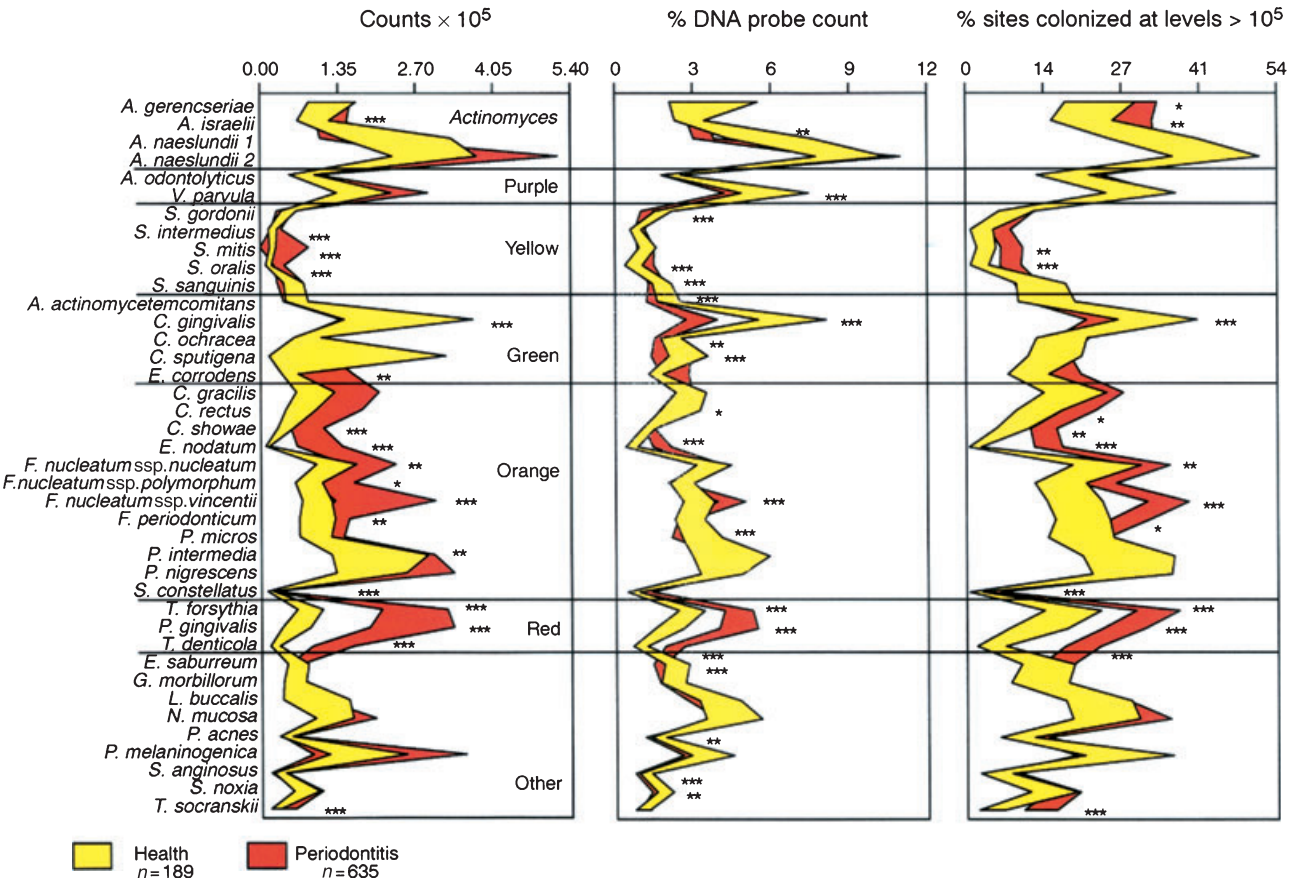

Fig. 1. Plots of mean counts (left panel), percentages of the total DNA probe count (middle panel) and percentage of sites colonized by 40 bacterial species at counts $>10^5$ (right panel) in subgingival plaque samples taken from 189 periodontally healthy subjects and 635 subjects with chronic periodontitis. The 'bands' represent the mean values ± the 95% confidence intervals after adjusting for 40 comparisons. Mean values for each species were computed by averaging up to 28 samples in each subject, and then averaging across subjects in the two clinical groups. Significance of differences between groups was sought using the non-parametric Mann–Whitney test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ after adjusting for multiple comparisons. The species were ordered and grouped according to the complexes described by Socransky et al. (79). The yellow profile represents the mean data for the healthy subjects and the red profile represents the data for the periodontitis subjects.

Percentage of subjects in each group that exhibited proportions of test species in the upper quartile

The percentage of periodontally healthy subjects and those with chronic periodontitis that exhibited proportions of the total DNA probe counts in the upper quartile for each species is presented in Fig. 3. *Eubacterium nodatum*, *T. forsythia*, *T. denticola*, *P. gingivalis*, *Streptococcus oralis* and *F. nucleatum* ssp. *vincentii* were found in significantly elevated proportions more commonly in subjects with periodontitis than in periodontally healthy subjects. *C. gingivalis*, *Streptococcus gordonii*, *Actinomyces naeslundii* genospecies 1, *P. melaninogenica*, *Capnocytophaga sputigena*, *A. actinomycetemcomitans*, *Leptotrichia buccalis*, *P. micros*, *Eubacterium saburreum*, *F. nucleatum* ssp. *polymorphum*, *Streptococcus anginosus* and *Streptococcus sanguinis* were found at elevated proportions significantly more often in healthy than diseased subjects.

Percentile plots of *P. gingivalis* and *T. forsythia* in the two clinical groups

Since *P. gingivalis* and *T. forsythia* are consensus pathogens of chronic periodontitis, the distributions of these species were examined in more detail in the two clinical groups (Fig. 4). There was a clear difference between the distributions of the mean percentages of the total DNA probe counts of the two species both individually and in combination between periodontally healthy subjects and subjects with chronic periodontitis. However, there was considerable overlap in the distributions. Indeed, as shown in the top panel, 28.8% (183) of the periodontitis subjects had *P. gingivalis* plus *T. forsythia* proportions lower than the median value (4.42%) for the periodontally healthy individuals. The question then became, in subjects with low proportions of the consensus pathogens, *P. gingivalis* and *T. forsythia*, which species differed significantly between health and disease?

Comparison of the subgingival microbiota in health and disease for subjects with low proportions of *P. gingivalis* and *T. forsythia*

Figure 5 presents the mean counts ($\times 10^5$) and the mean percentage of the total DNA probe count of the 40 test species in subgingival plaque samples from the 95 periodontally healthy and 183 chronic periodontitis subjects who had proportions of *P. gingivalis* plus *T. forsythia* less than the median value (4.42%) of the sum of these species in periodontally healthy subjects. Species that were significantly elevated in disease for both counts and proportions after adjusting for multiple comparisons were *S. oralis*, *E. corrodens*, *E. nodatum* and *T. denticola*. Mean counts

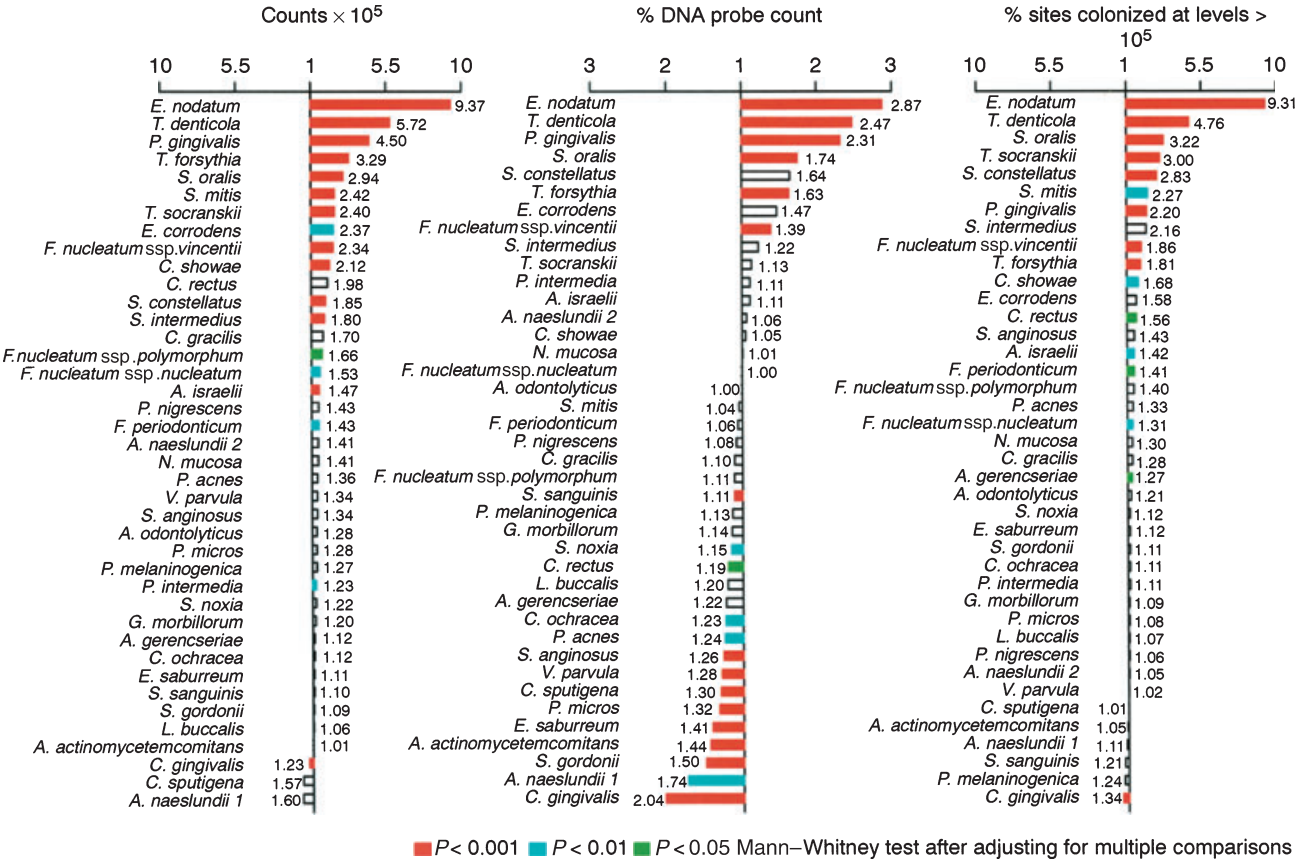

Fig. 2. Bar charts of the ratios of the mean counts, mean percentage of the total DNA probe counts and mean percentage of sites colonized at $>10^5$ by the 40 test species in samples from periodontally diseased and healthy subjects. Bars to the right of the vertical axis represent species with higher mean values in the diseased subjects, while bars to the left represent species with higher mean values in health. The numbers at the end of each bar represent the ratio of the mean values for the diseased to the healthy category. The colored bars represent the significances presented in Fig. 1.

of *Actinomyces israelii*, *Streptococcus intermedius*, *F. nucleatum* ssp. *vincentii*, *Streptococcus constellatus* and *P. gingivalis* were significantly higher in diseased than in healthy subjects. Mean counts and proportions of *C. gingivalis* as well as mean proportions of *S. gordonii*, *A. actinomycetemcomitans*, *Capnocytophaga ochracea*, *C. rectus*, *P. micros*, *T. forsythia* and *Selenomonas noxia* were significantly higher in health than disease.

Percentage of subjects with low proportions of *P. gingivalis* plus *T. forsythia* in periodontal health or disease that exhibited proportions of the 40 test species in the upper quartile

The percentage of subjects with low proportions of *P. gingivalis* plus *T. forsythia* who exhibited proportions of the total DNA probe counts in the upper quartile for the 40 test species is presented in Fig. 6. The periodontally healthy and chronic periodontitis subjects had proportions of *P. gingivalis* plus *T. forsythia* less than the median value (4.42%) of the sum of these species in periodontally healthy subjects. *E. nodatum*, *T. denticola* and *E. corrodens* were found in elevated proportions significantly more often in subjects with periodontitis than in periodontally healthy subjects. *C. gingivalis*, *S. gordonii*, *T. forsythia*, *S. noxia*, *A. actinomycetemcomitans* and *C. ochracea* were found at elevated proportions significantly more often in healthy than in diseased subjects.

Odds ratios of high proportions of 40 test species in periodontally healthy or diseased subjects who had low proportions of *P. gingivalis* and *T. forsythia*

The odds of periodontitis subjects and periodontally healthy subjects exhibiting percentages of the total DNA probe count in the upper quartile vs. the lower quartile of each species distribution was computed. Figure 7 presents the odds ratio (±95% confidence interval) for the 95 periodontally healthy and 183 chronic periodontitis subjects who had proportions of *P. gingivalis* plus *T. forsythia* less than the median value (4.42%) of the sum of these species in periodontally healthy subjects. The odds ratios showed that *E. nodatum*, *T. denticola*, *S. oralis*, *E. corrodens*, *S. intermedius* and *F. nucleatum* ssp. *vincentii* were in the upper quartile of the distribution significantly more often in diseased than periodontally healthy subjects. Conversely, the odds ratios of *C. gingivalis*, *S. gordonii*, *C. ochracea*, *S. noxia*, *T. forsythia*, *C. rectus*, *A. actinomycetemcomitans*, *Veillonella parvula*, *C. sputigena*, *P. micros*, *S. anginosus* and *Campylobacter showae* showed an association of high proportions of these taxa with periodontal health in this subset of subjects.

Logistic regression analysis

The relationship of combinations of predictor variables to the outcome variable, periodontal health or disease was examined, for all 824 subjects, using stepwise logistic regression analysis. The seven variables selected are presented in Table 2.

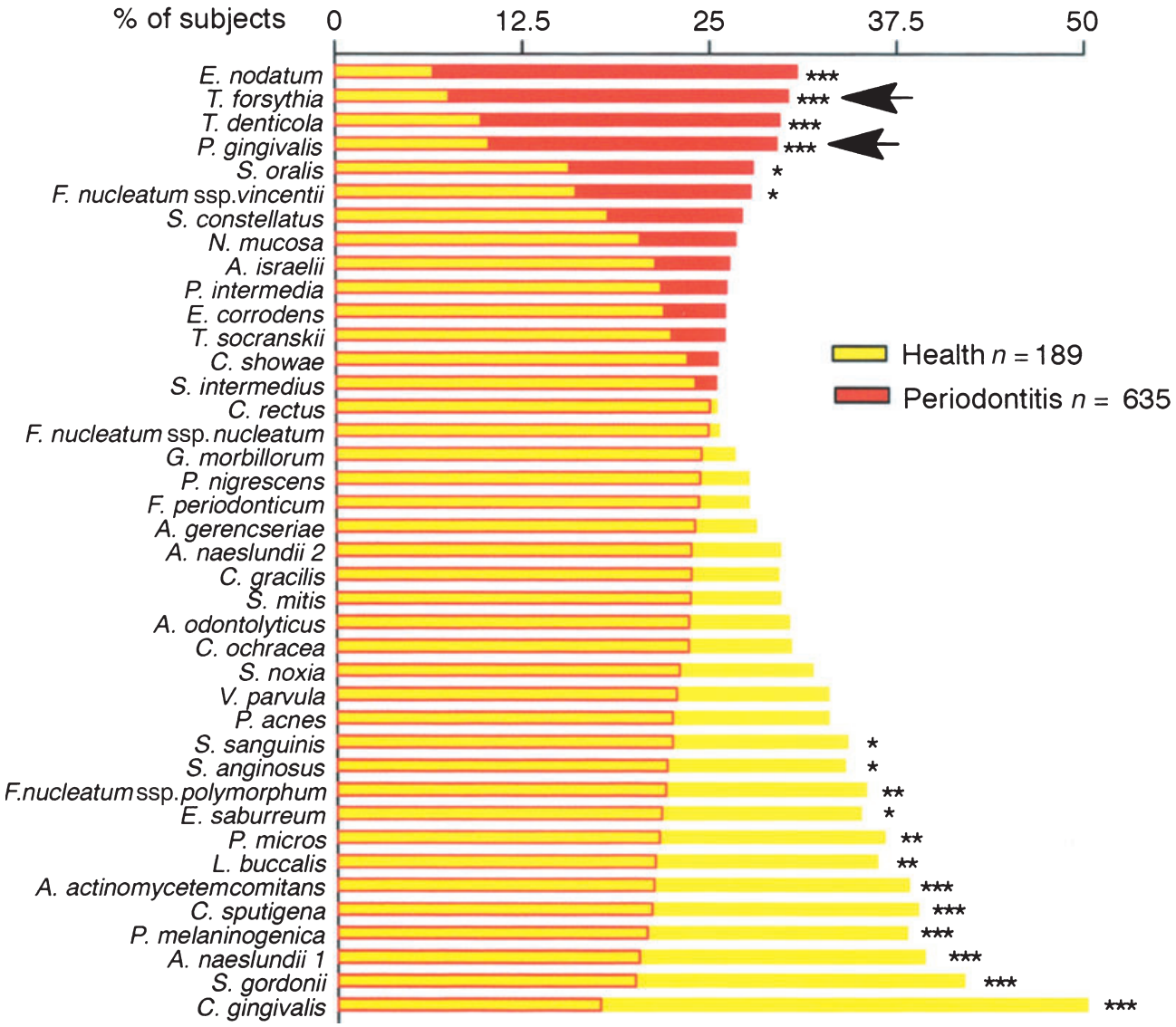

Fig. 3. Bar chart indicating the % of periodontally healthy and chronic periodontitis subjects who exhibited mean percentages of the total DNA probe counts of each test species that were in the upper quartile of counts for that species. For example, 30% of periodontitis subjects and 6% of periodontally healthy subjects exhibited counts that were above 1.79% of the total DNA probe count; i.e. the upper quartile encountered for *Eubacterium nodatum*. Significance of differences between clinical groups was sought using chi-squared test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ after adjusting for multiple comparisons. The arrows indicate the location of the 'consensus' periodontal pathogens, *Porphyromonas gingivalis* and *Tannerella forsythia*.

Variable selection terminated when the variable entering the model had a *P*-value >0.05. Table 2 summarizes the coefficients, estimated odds ratios, lower and upper 95% confidence intervals and probability values for each variable in the resulting model given the presence of the other six variables in the model. The relative odds associated with a unit change in each variable are presented. Thus, a 1% increase in the proportion of *E. nodatum* was associated with a relative odds of 1.77. Two subjects differing by 5% of *E. nodatum* with all other variables equal would have a relative odds of $(1.77)^5 = 17.37$. Similarly, a 1% difference in *E. nodatum* and a 1% difference in *T. denticola* would be the product of their separate odds ($1.77 \times 1.60 = 2.83$).

The analysis was repeated using data from the 278 subjects who had proportions of *P. gingivalis* plus *T. forsythia* lower than the median value (4.42%) observed in periodontally healthy subjects (Table 3). *E. nodatum*, *T. denticola*, *A. actinomycetemcomitans* and *S. gordonii* were selected in this model as well as in the model describing the full base of 824 subjects (Table 2). Species in the 278 subject analysis that were associated with disease included *E. nodatum*, *T. denticola*, *E. corrodens*, *Actinomyces naeslundii* 2 and *S. intermedius*. Species associated with health include *C. gingivalis*, *A. actinomycetemcomitans* and *S. gordonii*.

Discussion

The purpose of the present investigation was to seek species, other than the consensus periodontal pathogens, that were associated with chronic periodontitis. This was addressed by examining the association of 40 subgingival species with periodontal disease or health. The study confirmed the strong association of *P. gingivalis* and *T. forsythia* with chronic

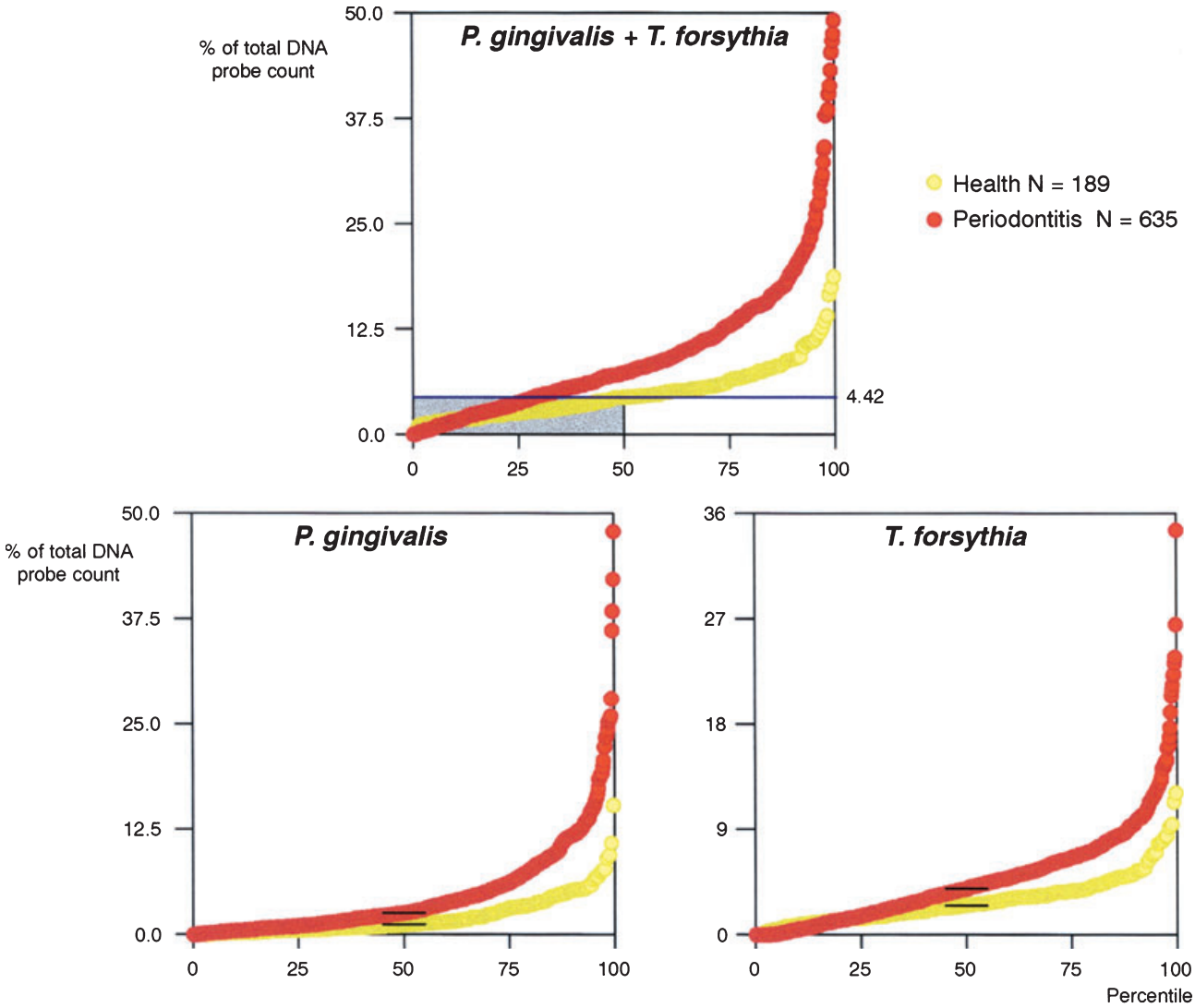

Fig. 4. Percentile plots showing the percentage of the total DNA probe counts of *Porphyromonas gingivalis* (bottom left panel), *Tannerella forsythia* (bottom right panel) and the sum of the two species proportions (top panel) in subjects placed in subsets according to periodontal health or disease. The *x*-axis represents the subject percentile and the *y*-axis represents the percentage of the DNA probe count. The percentage of the total DNA probe count was determined at each site for each species and then averaged within each subject for each species separately. Each circle depicts the mean percentage of the total DNA probe count for an individual subject. The short horizontal lines represent the median values (50th percentile) for each species for each clinical category. In each plot, subjects with low % total DNA probe count are reported to the left and subjects with increasing proportions of that species or combination of species are reported to the right. The horizontal line in the top panel represents the median value (4.42%) for the sum of the *P. gingivalis* + *T. forsythia* proportions in the periodontally healthy subjects. The gray-shaded area outlines the 95 periodontally healthy and 183 periodontitis subjects who had *P. gingivalis* + *T. forsythia* proportions below this value.

periodontitis whether evaluated by mean counts, mean proportions of the total DNA probe counts or percentage of sites colonized by species at counts $>10^5$. In addition, two other species, *E. nodatum* and *T. denticola*, were strongly associated with disease whether examining the full base of 824 subjects or a base of 278 subjects who exhibited lower proportions of *P. gingivalis* and *T. forsythia.* Other species were clearly health-related, including *C. gingivalis*, *S. gordonii*, *V. parvula*, *A. actinomycetemcomitans* and *A. naeslundii* genospecies 1.

E. nodatum appeared to be the species most strongly associated with chronic periodontitis both in the presence of high levels of *P. gingivalis* plus *T. forsythia* and in subjects where these species were in lower proportions. This species has been associated with periodontitis in other investigations. Moore and Moore (61) used the roll tube cultural technique to examine the proportions of bacterial species in subgingival plaque samples from subjects with various forms of periodontitis and gingivitis and in healthy subjects. They found that *E. nodatum* was absent or in low proportions in periodontal health and various forms of gingivitis, but was present in higher proportions in moderate periodontitis (2%), generalized early onset periodontitis (8%), localized juvenile periodontitis (6%), early onset periodontitis (5%) and adult periodontitis (2%). *E. nodatum* was in the top two to 14 species enumerated in these different periodontal states. More recent studies have confirmed an association of *E. nodatum* with periodontitis using molecular techniques. Using species-specific oligonucleotide probes, Booth et al. (10), investigated the relation-

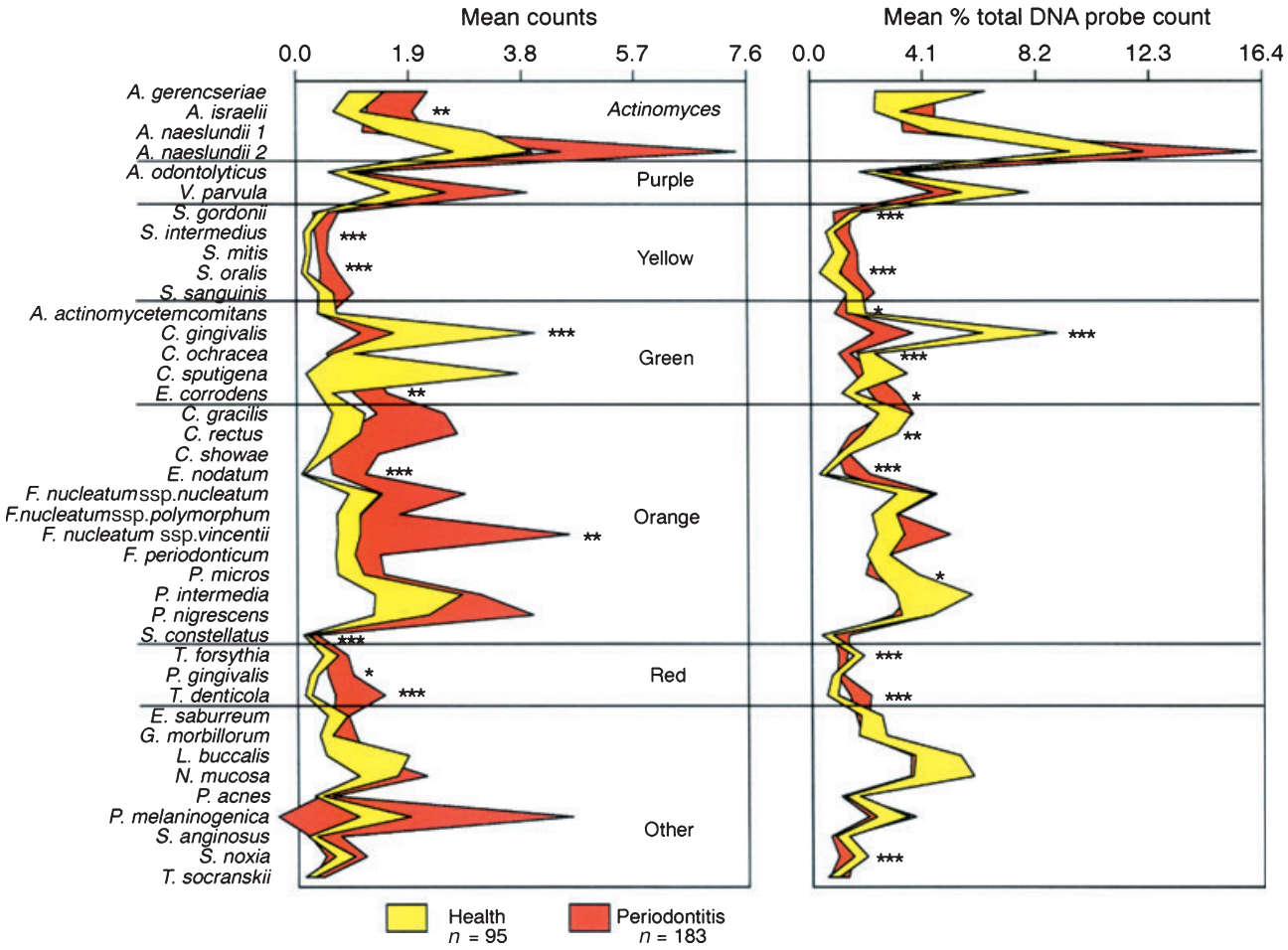

Fig. 5. Plots of mean counts and mean percentages of the total DNA probe count of 40 bacterial species in subgingival plaque samples taken from 95 periodontally healthy and 183 chronic periodontitis subjects who had *Porphyromonas gingivalis* + *Tannerella forsythia* percentage of total DNA probe counts less than the median value (4.42%) for periodontally healthy subjects. The 'bands' represent the mean values ±95% confidence intervals after adjusting for 40 comparisons. Computation of the 'bands' and explanation of significance of differences were as described for Fig. 1.

ship of selected gram-positive anaerobic bacilli to periodontal disease. They found that *E. nodatum* had significantly higher counts in patients than in matched control subjects. The species was also found at higher levels in deep pockets compared to shallow pockets. Papapanou et al. (66) found higher counts of *E. nodatum* in 131 periodontitis patients than in 74 periodontally intact controls using checkerboard DNA–DNA hybridization. Colombo et al. (18) also used checkerboard DNA–DNA hybridization to evaluate the microbiota in 25 untreated Brazilian subjects with chronic periodontitis and found a significant positive correlation of *E. nodatum* with mean pocket depth and attachment level. The percentage of sites colonized by *E. nodatum* was found to be significantly higher in current smokers than non-smokers (41).

Of the three red complex species (78), only *T. denticola* has not been formally designated a periodontal pathogen. However, the cumulative evidence available in the literature and the present investigation, strongly implicates this spirochete as an etiological agent of periodontal tissue destruction. *T. denticola* has been associated with chronic periodontitis in a number of studies (7, 38, 74, 85, 103). The level of *T. denticola*, determined using real-time PCR or checkerboard DNA–DNA hybridization has been found to correlate with pocket depth (4, 22, 102). Kigure et al. (48) and Noiri et al. (64) investigated the localization of this bacterium in the periodontal pocket using immunohistochemistry. In both studies it was demonstrated that *T. denticola* was localized in the 'unattached biofilm', in the middle and deep pocket zones. Studies evaluating periodontal therapy demonstrated an association between the reduction in the prevalence and/or levels of *T. denticola* and clinical improvement (20, 21, 36, 37, 99). Jin et al. (46) related changes in interleukin-8 (IL-8) and granulocyte elastase activity (MR-EA) in gingival crevicular fluid to the presence of subgingival periodontopathogens after anti-infective therapy in subjects with chronic periodontitis. In pockets ≥5 mm, the elimination of a co-infection with *T. forsythia*, *P. gingivalis*, *P. intermedia* and *T. denticola* was accompanied by significant reductions in the levels of IL-8, MR-EA and pocket depth. This effect was not observed when the co-infection persisted.

A large number of virulence factors have been described for *T. denticola* [for a comprehensive review, see Ellen & Galimanas (27)]. Briefly, factors described for *T. denticola* include: virulence mechanisms such as: induction of RANKL (15); production of dentilisin (a serine protease involved in epithelial penetration) (12); induction of IL-8 by an epithelial cell line (5); induction by lipo-oligosaccharides of

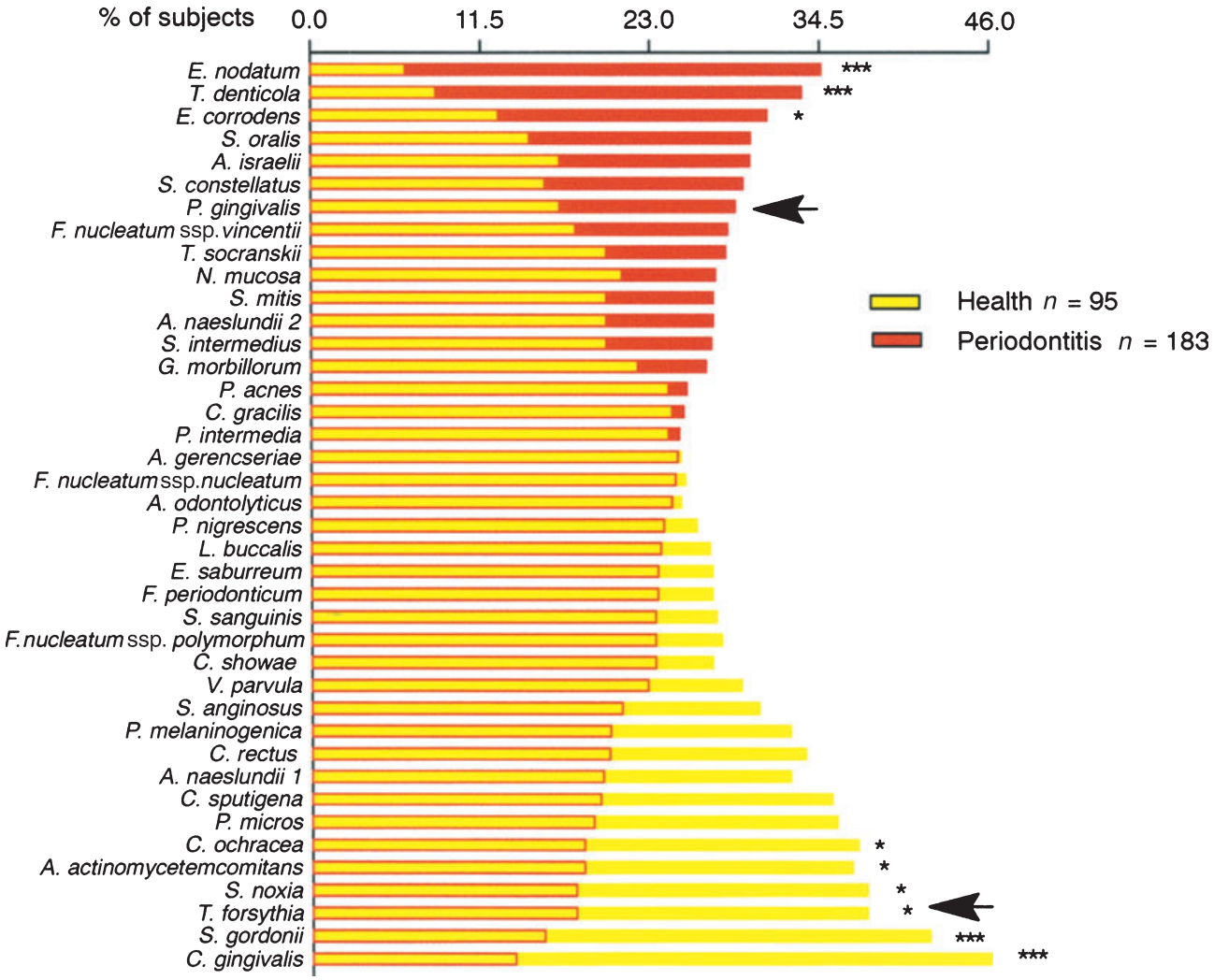

Fig. 6. Bar chart indicating the percentage of periodontally healthy or chronic periodontitis subjects who exhibited mean percentages of the total DNA probe counts of each test species that were in the upper quartile of counts for that species. The 95 periodontally healthy and 183 chronic periodontitis subjects had *Porphyromonas gingivalis* + *Tannerella forsythia* % total DNA probe counts less than the median value (4.42%) for periodontally healthy subjects. Significance of differences between groups was sought using chi-squared analysis; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ after adjusting for multiple comparisons. The arrows indicate the location of the 'consensus' periodontal pathogens, *P. gingivalis* and *T. forsythia*.

osteoclast maturation and activation and of the production of several matrix metalloproteinases (14); cytotoxicity for fibroblasts (92); release of a phenylalanine-specific protease (69); activation of murine macrophages through membrane-associated lipoproteins and lipo-oligosaccharides (70); activation of IL-1β precursor (9); modulation of neutrophil oxygen radical production (75); and degradation of host protease inhibitors (34).

Perhaps the most compelling evidence of a cause–effect association between a microorganism and periodontal infections is provided by risk assessment studies. Papapanou et al. (65) demonstrated, that certain specific pathogens were associated with deep pockets and progressing sites in 1864 samples from 148 Chinese subjects. Colonization by *T. denticola* (as well as other putative pathogens), above certain thresholds, increased significantly the odds of a subject exhibiting periodontal disease progression. The levels and prevalence of *T. denticola* were also reported to be associated with disease progression in a group of patients with early onset periodontitis (2). Finally, an elevated immunoglobulin G response specific for *T. denticola* has been reported in patients with chronic periodontitis (50) and periodontal treatment resulted in a decrease in the titers of serum antibodies to this species (50).

In some of the analyses in the current investigation, other species were found to be associated with periodontitis, particularly in the subjects with low levels of *P. gingivalis* and *T. forsythia*, including *S. intermedius*, *S. oralis* and *E. corrodens*. Although the literature describing an association of these species with periodontitis is limited, *S. intermedius* has been associated with cases of Papillon–Lefevre syndrome (55), refractory periodontitis (58) and was a prominent species detected in IL-1 genotype-positive chronic periodontitis patients (80). Furthermore, hyaluronidase activity has been detected in human pus samples from which the only recovered isolate was *S. intermedius* as well as in culture supernatants of this species (84). *S. oralis*, on occasion, has been described as a periodontal health-associated species (86), although an interaction between a coadhesin of *S. oralis* and *P. gingivalis* fimbriae has been demonstrated (57).

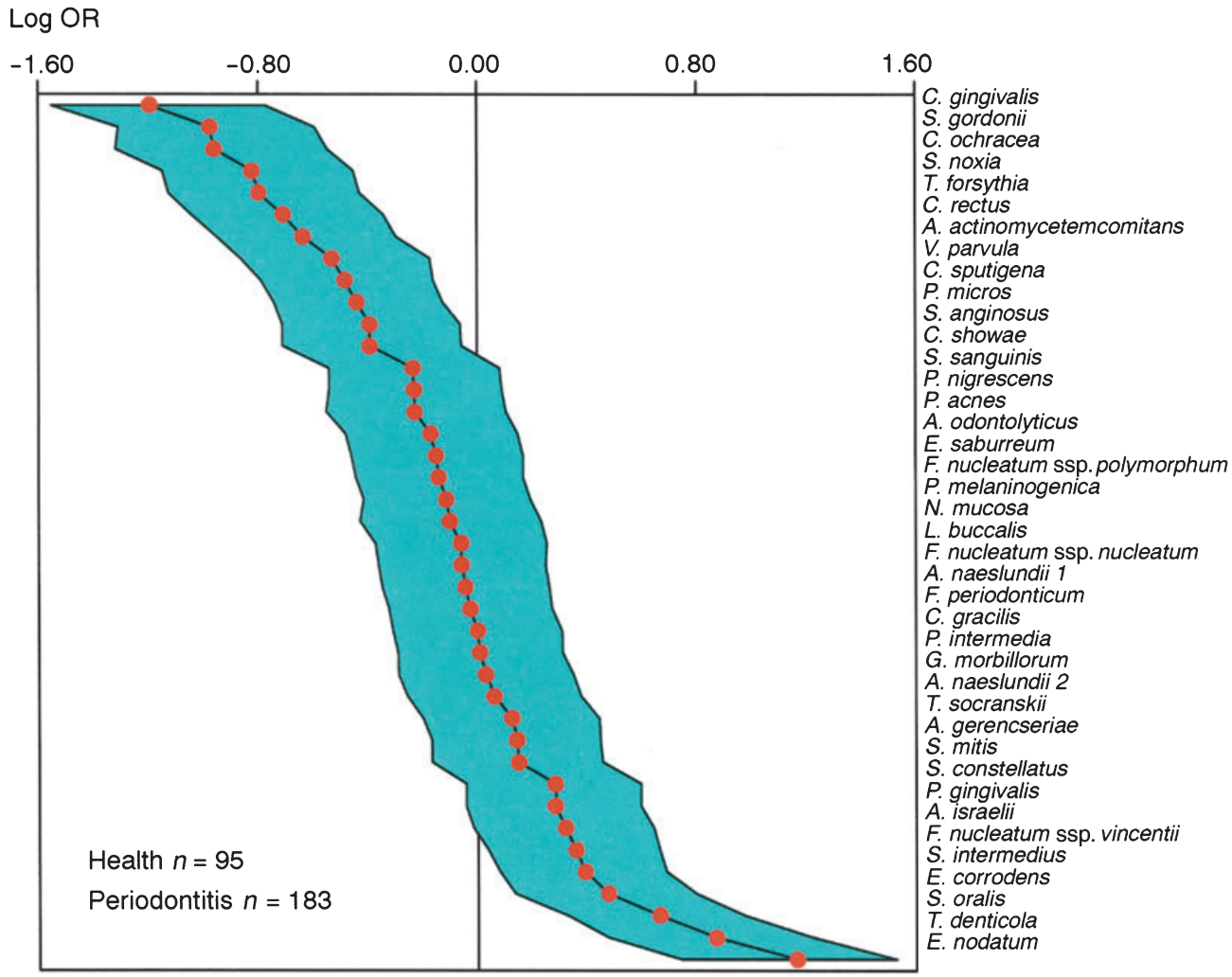

Fig. 7. Odds ratios and 95% confidence intervals of species in the upper quartile when compared with the lower quartile of the distribution in periodontally healthy and diseased subjects. The 95 periodontally healthy and 183 chronic periodontitis subjects had *P. gingivalis* + *T. forsythia* percentage of total DNA probe counts less than the median value (4.42%) for periodontally healthy subjects.

Table 2. Logistic regression analysis of mean percentage of the total DNA probe count of species in the 189 periodontally healthy and 635 chronic periodontitis subjects

Variable	Coefficient	Odds ratio	95% CI		*P*-value
			Lower	Upper	
Constant	1.0734				
Eubacterium nodatum	0.5720	1.7719	1.3676	2.2957	<0.001
Treponema denticola	0.4713	1.6021	1.2817	2.0026	<0.001
Actinobacillus actinomycetemcomitans	−0.2330	0.7921	0.7077	0.8866	<0.001
Tannerella forsythia	0.2162	1.2414	1.1385	1.3535	<0.001
Streptococcus gordonii	−0.4154	0.6600	0.5640	0.7725	<0.001
Actinomyces naeslundii genospecies 1	−0.1095	0.8963	0.8517	0.9432	<0.001
Veillonella parvula	−0.0731	0.9295	0.8892	0.9716	<0.001

The model was arrived at by forward stepwise regression. $\text{Log}_e(P/1-P) = 1.0734 + 0.5720$ *E. nodatum* + 0.4713 *T. denticola* − 0.2330 *A. actinomycetemcomitans* + 0.2162 *T. forsythia* − 0.4154 *S. gordonii* − 0.1095 *Actinomyces naeslundii*-1 − 0.0731 *V. parvula*.
Chi-square = 232.9 with 7 d.f., $P < 0.000001$.

E. corrodens has been found to be associated mainly with cases of aggressive or early onset periodontitis (13, 29, 31, 82), but has also been implicated in adult, chronic forms of the infection (7, 62, 103). In recent years, several virulence factors produced by this species have been described. Yamada et al. (100) demonstrated that a human epithelial cell line (KB) could be induced *in vitro* to express intercellular adhesion molecule-1 after exposure to *E. corrodens* adhesins. Both the cells and the supernatant of *E. corrodens* can also induce KB cells to produce IL-6, IL-8 and prostaglandin E_2 (104, 105). Furthermore, *E. corrodens* can produce a lysine decarboxylase (p80), capable of inhibiting mammalian cell growth by depriving the cells of lysine (52).

A number of analytical issues arose while examining the data from this investigation. One of the problems when comparing microbiological data in periodontal health and disease is how to present the findings. Should the levels or counts of individual species, the proportion that each species makes up of the total DNA probe count or, if sufficient teeth have been sampled, the prevalence or percentage of

Table 3. Logistic regression analysis of mean percentage of the total DNA probe count of species in 95 periodontally healthy and 183 chronic periodontitis subjects

Variable	Coefficient	Odds ratio	95% CI Lower	95% CI Upper	*P*-value
Constant	−1.0841				
Eubacterium nodatum	0.9443	2.5711	1.5469	4.2732	0.00001
Capnocytophaga gingivalis	−0.0543	0.9471	0.8957	1.0016	0.015
Actinobacillus actinomycetemcomitans	−0.2102	0.8104	0.6611	0.9933	0.039
Treponema denticola	0.4531	1.5733	1.1054	2.2392	0.002
Streptococcus gordonii	−0.1899	0.8270	0.6916	0.9890	0.038
Eikenella corrodens	0.2262	1.2538	1.0232	1.5365	0.008
Actinomyces naeslundii genospecies 2	0.0479	1.0491	1.0100	1.0896	0.004
Streptococcus intermedius	0.3760	1.4564	1.0339	2.0517	0.019

These subjects were selected on the basis of having mean % *P. gingivalis* + *T. forsythia* < 4.24% (the mean value for periodontally healthy subjects). A model was arrived at by forward stepwise regression.

$\text{Log}_e(P/1-P) = -1.0841 + 0.9443$ *E. nodatum* $- 0.0543$ *Capnocytophaga gingivalis* $- 0.2102$ *A. actinomycetemcomitans* $+ 0.4531$ *T. denticola* $- 0.1899$ *S. gordonii* $+ 0.2262$ *E. corrodens* $+ 0.0479$ *Actinomyces naeslundii*-2 $+ 0.3760$ *S. intermedius*.

Chi-square = 105.6 with 8 d.f., $P < 0.000001$.

sites colonized be presented? All three methods were employed to examine the microbial differences between health and disease for the 824 subjects. The three presentations showed similar trends with many species being significantly elevated in disease compared with health in counts, proportions and percentage of sites colonized at levels $>10^5$. In particular, all three red complex species, and *S. oralis*, *E. nodatum* and *F. nucleatum* ssp. *vincentii* were significantly elevated in periodontitis compared to health and *C. gingivalis* was significantly higher in healthy subjects by all three descriptors. However, to spare the reader wading through endless figures presenting counts, percentages and prevalences, a single method of presentation was used for subsequent analyses. The % DNA probe count was selected because it is the most conservative of the data presentations and is least sensitive to the higher total bacterial counts typically observed in subjects with periodontitis compared with periodontally healthy subjects.

A second issue was when to consider the periodontitis subjects as having low levels or proportions of the consensus pathogens, *P. gingivalis* and *T. forsythia.* There was no simple guideline for this decision. We chose as the threshold the median (4.42%) of the sum of the % of the total DNA probe counts of the two species combined in the periodontally healthy subjects. It was reasoned that if periodontitis subjects had proportions of these species at or below the levels found in half of the periodontally healthy subjects, they could justifiably be thought of as having low proportions of these species. Additional thresholds ranging from the 20th percentile to the 60th percentile of counts in periodontally healthy subjects gave essentially the same results (data not shown).

As in every study, the present investigation had certain strengths and limitations. The major strengths were the number of subjects sampled, 183 periodontally healthy and 625 periodontitis subjects, the number of plaque samples examined (21,832 samples or a mean of 26.5 per subject), as well as the wide range of species evaluated (40). The data represented over 750,000 bacterial counts. Thus, this study may represent the most comprehensive comparison of periodontal health and disease reported to date. The major limitation of this study was that not all species that can inhabit the subgingival area were evaluated. While the 40 DNA probes employed accounted for about 50–55% of the subgingival plaque biomass (77), it is recognized that an additional 400–500 species may be encountered in this habitat although many of these taxa may be in low numbers and infrequently encountered (68, 77). A second limitation is that the subject population was derived from only two communities, one in the USA and one in Sweden. It is recognized that the subgingival microbiota in periodontitis (35) and periodontal health (39) differs in subjects from different geographic locations.

A third limitation of this study was that the DNA probes employed could not distinguish between virulent and avirulent clonal types of pathogenic species. Perhaps the most intriguing example of this limitation is provided by the data for *A. actinomycetemcomitans.* In the present study, *A. actinomycetemcomitans* levels and proportions were both significantly higher in subjects who were periodontally healthy than in the chronic periodontitis subjects. However, *A. actinomycetemcomitans* is considered to be a pathogen of certain aggressive forms of periodontitis, including localized aggressive periodontitis (for review see refs 40, 106) and perhaps certain cases of refractory periodontitis (91). It is recognized that highly leukotoxic strains of *A. actinomycetemcomitans* are associated with aggressive forms of periodontitis (45). However, many, perhaps most, subjects are colonized by less pathogenic strains of *A. actinomycetemcomitans*. Indeed, *A. actinomycetemcomitans* can be found in subgingival plaque samples from periodontally healthy subjects and is often in high numbers in supragingival plaque and on soft tissues (63) as well as in buccal epithelial cells (71). Thus, a probe distinguishing virulent and avirulent clonal types would be useful in clarifying the role of this species in different forms of periodontitis and, in particular, it might distinguish chronic periodontitis subjects in whom the species might play an etiological role.

The present investigation confirmed the strong association of *P. gingivalis* and *T. forsythia* with chronic periodontitis. It also emphasized the strong association of *E. nodatum* and *T. denticola* with this form of disease whether in the presence or absence of high levels or proportions of the two consensus periodontal pathogens. When *P. gingivalis* and *T. forsythia* were present in low proportions in a subset of subjects, other species such as *S. oralis*, *E. corrodens*, *S. intermedius* and *F. nucleatum* ssp. *vincentii* were also present in higher counts and or proportions in subjects with chronic periodontitis than in subjects who were periodontally healthy. Studies of the association of a microbial species with disease are only the first step in developing the chain of evidence necessary to define periodontal pathogens but such studies are the essential first step. The results of this investigation suggest taxa that warrant additional study in terms of virulence properties, host response, risk assessment, animal model testing and studies of the effect of the elimination/suppression of the species on disease progression.

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