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Differences in the subgingival microbiota of Swedish and USA subjects who were periodontally healthy or exhibited minimal periodontal disease

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Abstract

Background: Previous studies have shown differences in the mean proportions of subgingival species in samples from periodontitis subjects in different countries, which may relate to differences in diet, genetics, disease susceptibility and manifestation. The purpose of the present investigation was to determine whether there were differences in the subgingival microbiotas of Swedish and American subjects who exhibited periodontal health or minimal periodontal disease.

Method: One hundred and fifty eight periodontally healthy or minimally diseased subjects (*N* Sweden = 79; USA = 79) were recruited. Subjects were measured at baseline for plaque, gingivitis, BOP, suppuration, pocket depth and attachment level at 6 sites per tooth. Subgingival plaque samples taken from the mesial aspect of each tooth at baseline were individually analyzed, in one laboratory, for their content of 40 bacterial species using checkerboard DNA–DNA hybridization (total samples = 4345). % DNA probe counts comprised by each species was determined for each site and averaged across sites in each subject. Significance of differences in proportions of each species between countries was determined using ANCOVA adjusting for age, mean pocket depth, gender and smoking status. *p* values were adjusted for multiple comparisons. Cluster analysis was performed to group subjects based on their subgingival microbial profiles using a chord coefficient and an average unweighted linkage sort.

Results: On average, all species were detected in samples from subjects in both countries. After adjusting for multiple comparisons, 5 species were in significantly higher adjusted mean percentages in Swedish than American subjects: *Actinomyces naeslundii* genospecies 1 (9.7, 3.3); *Streptococcus sanguis* (2.5, 1.2); *Eikenella corrodens* (1.7, 1.0); *Tannerella forsythensis* (3.5, 2.3) and *Prevotella melaninogenica* (6.3, 1.8). *Leptotrichia buccalis* was in significantly higher adjusted mean percentages in American (5.5) than Swedish subjects (3.0). Cluster analysis grouped 121 subjects into 8 microbial profiles. Twenty four of the 40 test species examined differed significantly among cluster groups. Five clusters were dominated by American subjects and 2 clusters by Swedish subjects. Fifty eight of 79 (73%) of the Swedish subjects fell into 1 cluster group dominated by high proportions of *A. naeslundii* genospecies 1, *Prevotella nigrescens, T. forsythensis* and *P. melaninogenica*. Other clusters were characterized by high proportions of *Actinomyces gerencseriae, Veillonella parvula, Capnocytophaga gingivalis, Prevotella intermedia, Eubacterium saburreum, L. buccalis* and *Neisseria mucosa*.

Conclusions: The microbial profiles of subgingival plaque samples from Swedish and American subjects who exhibited periodontal health or minimal disease differed. The heterogeneity in subgingival microbial profiles was more pronounced in the American subjects, possibly because of greater genetic and microbiologic diversity in the American subjects sampled.

Key words: biofilm; geographic location; periodontal health; plaque; subgingival microbiota

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In a previous study (Haffajee et al. 2004), it was found that the mean subgingival microbial profiles differed markedly in subjects enrolled in studies of the treatment of chronic periodontitis in Brazil, Chile, Sweden and the USA. Such differences in the subgingival microbiota could also have been deduced from other studies of the subgingival microbiota, although most of these studies did not directly compare the microbial samples originating from different countries in the same laboratory (for reviews, see Sanz et al. 2000, Haffajee et al. 2004). A natural extension of this finding was whether such differences in the composition of the subgingival microbiota would be observed in subjects who were periodontally healthy or exhibited minimal periodontal disease. Thus, the purpose of the present investigation was to compare the subgingival microbiotas in Swedish and American subjects enrolled in studies of the prevention of periodontal disease in healthy and minimally diseased subjects.

Material and Methods

Subject population

For the study 158 periodontally healthy or minimally diseased adult subjects who were enrolled in prevention studies in Landskrona, Sweden ($N = 79$) and Boston, USA ($N = 79$) were selected. Human Investigational Review Boards at the 2 collaborating institutions approved the protocol, including the taking of clinical measurements and plaque samples. All subjects were informed of the nature, potential risks and benefits of study participation and signed informed consent prior to entry into the study. Included subjects were $\geqslant 20$ years of age and had at least 24 natural teeth. Periodontally healthy subjects exhibited no pockets > 4 mm or attachment level measurements > 3 mm. Minimally diseased subjects had no more than 2 sites with pocket depth > 4 mm or 2 sites with attachment level measurements > 4 mm. Exclusion criteria included pregnancy, nursing, periodontal therapy and antibiotic administration within the previous 6 months as well as any systemic condition which might have affected periodontal status. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. Demographic parameters as well as smoking histories were obtained using a questionnaire. Members of the study teams reviewed all answers with the subject. The baseline clinical parameters for the subject groups in the 2 countries are presented in Table 1.

Clinical measurements

Measures of plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (BOP, 0/1), suppuration (0/1), probing pocket depth and probing attachment level were taken at 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars at a baseline visit. Pocket depth and attachment level measurements were made using a North Carolina probe.

Microbiological assessment

Subgingival plaque samples were taken at baseline from the mesiobuccal aspect of all teeth (excluding third molars). Samples were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. All samples were analyzed in the same periodontal laboratory at The Forsyth Institute. Counts of 40 subgingival species were determined in each plaque sample using a modification (Haffajee et al. 1997) of the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994). 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 mM 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 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 of 40 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA),

Table 1. Mean (±SD) clinical parameters of the Swedish and American subjects

	Sweden	USA	Mann-Whitney (p)
N	79	79	
Age (years)	37 ± 9	35 ± 11	$p < 0.05$
Number of missing teeth	0.7 ± 1.2	0.6 ± 1.4	ns
% Males	39	32	ns
% Sites with:			
plaque	21 ± 15	43 ± 18	$p < 0.001$
gingival redness	18 ± 13	34 ± 19	$p < 0.001$
BOP	32 ± 15	9 ± 7	$p < 0.001$
suppuration	0 ± 0	0 ± 0	ns
mean PD (mm)	2.48 ± 0.21	2.18 ± 0.25	$p < 0.001$
mean AL (mm)	2.52 ± 0.23	1.70 ± 0.56	$p < 0.001$
% Current smokers	24	9	$p < 0.05$

BOP, Bleeding on probing; PD, Pocket depth.

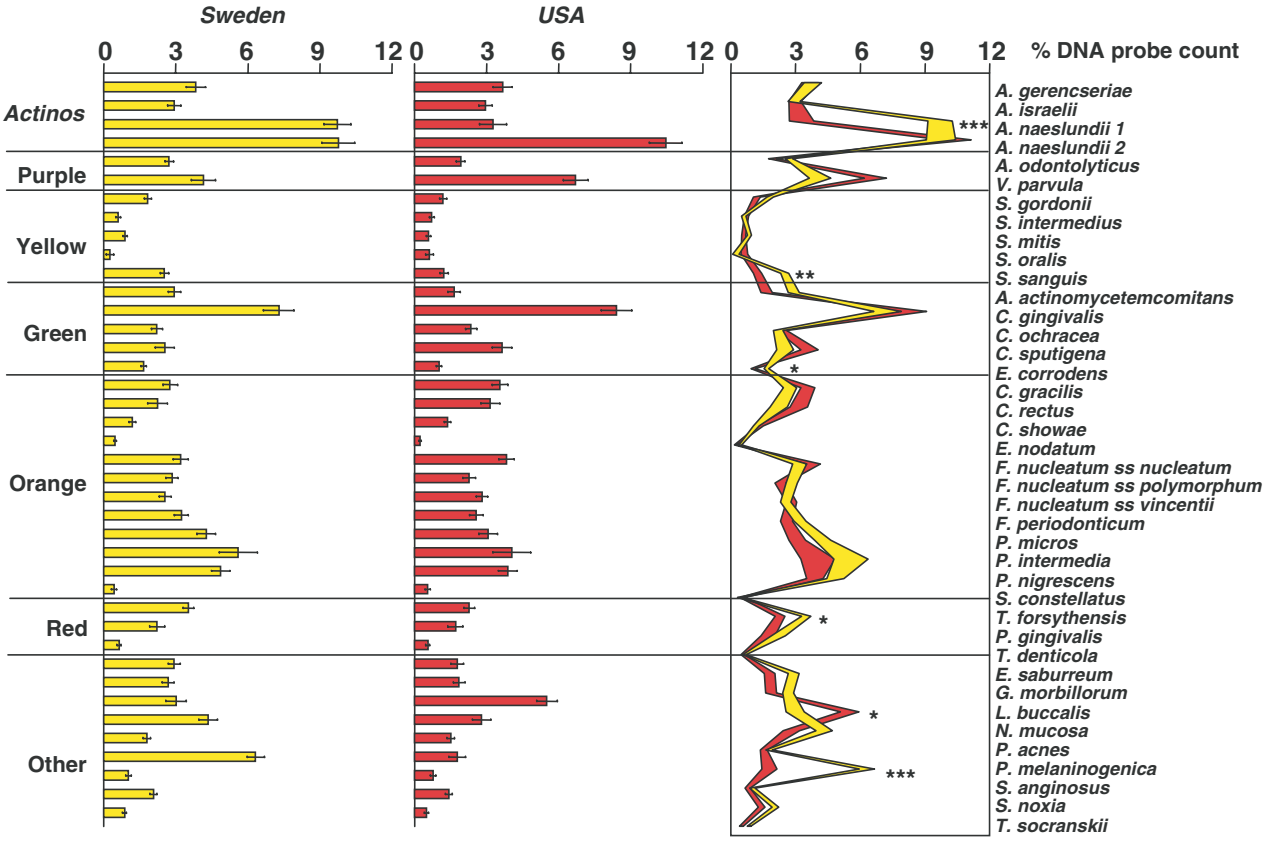

Fig. 1. Bar charts of adjusted mean percents (± SEM) of the total DNA probe count of 40 bacterial species in baseline subgingival plaque samples taken from 79 Swedish and 79 American periodontally healthy or minimally diseased subjects. The bars (left and middle panels) represent the mean percents after adjusting for age, mean pocket depth, gender and smoking status. The whiskers indicate the adjusted standard error of the mean. Mean percents of each species were computed by averaging up to 28 samples in each subject, and then averaging across subjects in the 2 countries. Significance of differences between groups was sought using ANCOVA adjusting for age, mean pocket depth, gender and smoking status; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ after adjusting for multiple comparisons. The species were ordered and grouped according to the complexes described by Socransky et al. (1998). In order to facilitate country-to-country species comparisons, the right panel presents the microbial profiles as ''bands'' in which the width of the band is depicted as the adjusted means ± SEMs of each species in samples from each country. The yellow profile represents the mean data for the Swedish subjects and the red profile represents the adjusted mean data for the American subjects.

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 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 4345 subgingival samples were evaluated for an average of 27.3 samples per subject.

Data analysis

The percentage of the total DNA probe count was determined for each species at each site in each subject and averaged within subjects in the different geographic locations. Significance of differences for each species across geographic locations was sought using ANCOVA, adjusting for age, mean pocket depth, gender and smoking status. *p* values were adjusted for multiple comparisons as described previously (Socransky et al. 1991). Cluster analysis was performed to group subjects based on the mean proportions of subgingival species using the chord coefficient (Ludwig & Reynolds, 1988) and an average unweighted linkage sort (Sneath & Sokal, 1973).

Results

Fig. 1 presents the adjusted mean % DNA probe count of the 40 test species in the Swedish and American subjects. On average, all species were detected in samples from subjects in both countries. The majority of species did not differ significantly in adjusted mean proportions from country to country. After adjusting for multiple comparisons, 5 species were in significantly higher adjusted mean percentages in Swedish than American subjects: *Actinomyces naeslundii* genospecies 1, 9.7, 3.3; *Streptococcus sanguis*, 2.5, 1.2; *Eikenella corrodens*, 1.7, 1.0; *Tannerella forsythensis*, 3.5, 2.3 and *Prevotella melaninogenica*, 6.3, 1.8; *Leptotrichia buccalis* was in significantly higher adjusted mean percentages in American (5.5) than is Swedish subjects (3.0).

Two species that differed significantly in mean counts between countries were examined further. Fig. 2 presents the mean counts of *A. naeslun-*

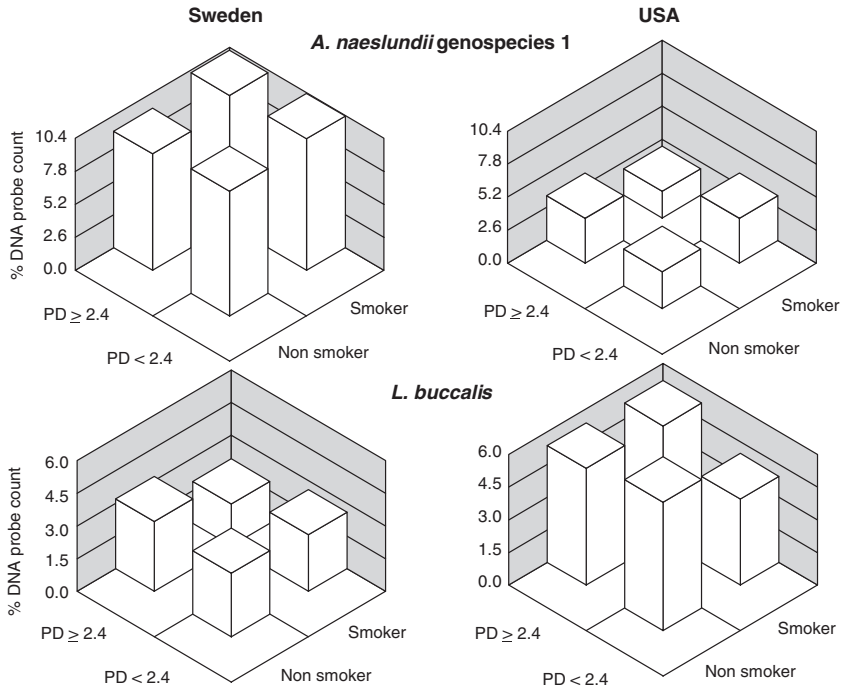

Fig. 2. Bar charts of mean proportions of *A. naeslundii* genospecies 1 (upper panels) and *L. buccalis* (lower panels). In American and Swedish subjects separately, the counts of the species were averaged in each subject, then averaged across subjects in groups categorized as current smoker or non-smoker, and with or without mean full mouth pocket depth $\geqslant$2.4 mm. The mean pocket depth value of 2.4 mm was the median for the entire subject group.

dii genospecies 1 and *L. buccalis* in Swedish and American subjects who were subset according to smoking status and mean full mouth pocket depth of $\geqslant$2.4 mm (median for subject population). The data confirmed the notion that differences in mean pocket depth or smoking status for subjects in the 2 countries had less effect on the mean bacterial counts than the geographic location of the sampled subject.

Cluster analysis was performed to group subjects according to their mean microbial profiles (Fig. 3). One hundred and twenty one of 158 subjects were assigned to 8 clusters at $>$48% similarity, while 37 subjects were not assigned to any cluster group. The number of subjects in each cluster group differed, ranging from 3 subjects in Cluster group 3 to 65 subjects in Cluster group 5. Five clusters and the subjects ''not in cluster'' were made up predominantly of American subjects. Clusters 3 and 7 were made up totally of American subjects. The largest group, Cluster 5, was dominated by Swedish subjects who comprised 90% (58 of 65 subjects). The 58 Swedish subjects represented 73% of Swedish subjects studied.

The mean microbial profiles of the subjects in the different cluster groups and ''not in cluster'' are presented in Fig. 4. Among cluster groups 24 of the 40 test species examined differed significantly even after adjusting for multiple comparisons. Swedish subjects were predominant in Clusters 1 and 5. Subjects in Cluster 5 exhibited the highest proportions of *A. naeslundii* genospecies 1 and *P. melaninogenica.* Cluster 1 was dominated by high proportions of *Actinomyces gerencseriae*, and *P. melaninogenica*. The clusters predominated by American subjects presented with high proportions of *Actinomyces naeslundii* genospecies 2. In addition, there were specific differences among cluster groups predominated by American subjects. Cluster 3 was dominated by high mean proportions of *Eubacterium saburreum,* Cluster 4 by *Neisseria mucosa*, Cluster 6 by *Capnocytophaga gingivalis* and *L. buccalis*, Cluster group 7 by *Veillonella parvula* and Cluster group 8 by *Prevotella intermedia.* All cluster groups and the ''not in cluster'' group had mean proportions of red complex species that were $<$4% of the microbiota.

Discussion

The present investigation evaluated the microbial composition of subgingival plaque samples taken from periodontally healthy or minimally diseased subjects in Sweden and the USA. The results indicated that there were differences in the patterns of colonization of the 40 test species between the 2 countries and also there was heterogeneity among subjects in both geographic locations. Overall, there were significantly higher adjusted mean proportions of *A. naeslundii* genospecies 1, *S. sanguis*, *E. corrodens*, *T. forsythensis* and *P. melaninogenica* in the Swedish subjects, while *L. buccalis* was significantly elevated in adjusted mean proportions in the American subjects. The finding of differences in the mean subgingival microbiota in periodontally healthy or minimally diseased subjects from different geographic locations was in agreement with similar findings of differences in the mean subgingival microbial profiles in chronic periodontitis subjects recruited in different countries (Haffajee et al. 2004). The extent to which these differences occur is not clear. It is conceivable that there may be major differences in the mean subgingival microbial profiles in subjects with a selected periodontal status who reside in different communities within the same country or even the same state. However, this type of comparison has not been carried out.

Cluster analysis was performed in order to examine differences and similarities in the microbial profiles among subjects. Eight distinct clusters were formed based on the subjects' subgingival plaque composition indicating diversity in the subgingival microbiota of periodontally healthy or minimally diseased subjects. Thus, no single microbial profile was associated with no or low levels of periodontitis, rather these subjects exhibited a wide range of quite distinct subgingival microbiotas. It seems likely that some of the microbial profiles may confer a greater risk for periodontal disease initiation than others. In addition, different microbial communities may predispose to different forms of periodontal disease. Differences in subgingival microbial composition among subjects with refractory or non-refractory periodontitis have already been reported (Socransky et al. 2002).

Certain subjects, however, did exhibit similar microbial profiles within each country and even between countries, as demonstrated by their inclusion in a given cluster. Cluster 5 provided the most striking example of this phenom-

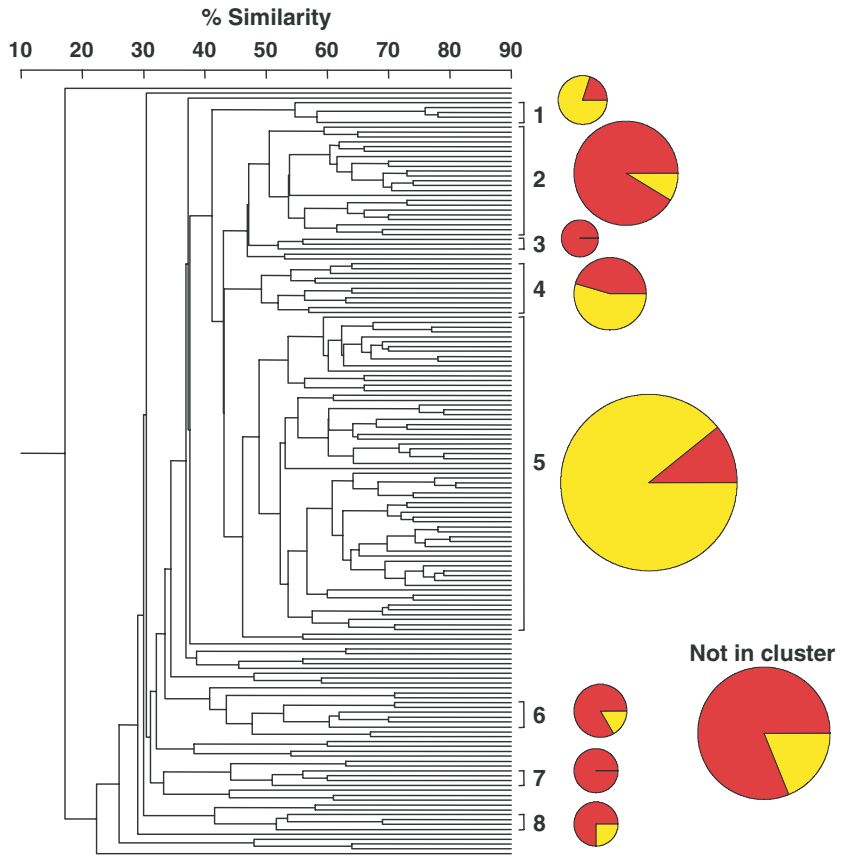

Fig. 3. Dendrogram of a cluster analysis based on the composition of the subgingival microbiota in samples taken from 79 Swedish and 79 American periodontally healthy or minimally diseased subjects. The mean percent of the total DNA probe count of each species was computed at each of (up to) 28 sites in each subject and averaged within a subject. The percentage similarities of the microbiotas among subjects were computed using the chord similarity coefficient and the resulting values were sorted using an average unweighted linkage sort. The pies represent the proportion of Swedish and American subjects in each cluster. The areas of the pies have been adjusted to reflect the total number of subjects in each cluster. The yellow area represents Swedish subjects and the red area represents the American subjects.

enon. This cluster group of 65 individuals was the largest and included 58 of the 79 Swedish subjects (73%). The subgingival microbiota of subjects in this cluster was dominated by high proportions of *A. naeslundii* genospecies 1 which may account for the high overall mean proportion of this species in the Swedish subjects. The subjects in this cluster also exhibited the highest adjusted mean proportions of *T. forsythensis, P. melaninogenica* and *P. nigrescens*. A second cluster (Cluster 1) that was dominated by Swedish subjects was different in mean microbial profile from Cluster 5. Subjects in this cluster exhibited lower proportions of *A. naeslundii* genospecies 1 and higher proportions of *A. gerencseriae*. The American subjects dominated 5 clusters and the ''not in cluster'' group indicating greater heterogeneity of their subgingival microbiotas compared with the Swedish subjects. These findings suggest greater microbial heterogeneity in the American than the Swedish populations from which the subjects were recruited. The subjects from the Boston area were racially and ethnically diverse. There were 62% white, 8.9% African American, 17.7% Asian, 7.6% Hispanic and 3.8% ''unsure'' individuals in the American group. On the other hand, the periodontally healthy subjects from Sweden were recruited from Landskrona, a small town south of Gothenburg, with arguably a more homogeneous subject population in terms of race, ethnicity, socio-economic status and cultural background. Indeed, all of the Swedish subjects were white, Caucasians of middle class background. When the ANCOVA comparing only white subjects in both countries was performed, similar statistically significant differences still existed between the microbial profiles in the samples from the 2 countries (data not shown). This suggests that racial/ethnic background was not the major source of the observed differences in the subgingival microbiota.

There were some limitations to this investigation. The subjects were not randomly selected and may not be representative of the ''country of origin''. There were small differences in baseline clinical and demographic features between the subjects from the 2 countries which may not have been adequately adjusted for in the analyses. However, inspection of the effect of the clinical and demographic parameters on the species that differed significantly between countries supported the notion that the major factor influencing the microbial differences was the country of origin (e.g. Fig. 2). In spite of these reservations, it was clear that the subgingival microbiotas in the sampled subjects differed between the 2 countries. Significant differences in the subgingival microbiota were still detected if there was no adjustment for baseline clinical and demographic parameters, or adjustment included all such parameters (data not shown). A major strength of the study was that the analysis of all plaque samples was performed in the same laboratory in the Department of Periodontology at the Forsyth Institute in Boston, eliminating the possibility of technical differences in microbial enumeration.

An additional strength of the present investigation was that it constituted a comprehensive evaluation of the composition of the subgingival microbiota in a large number of samples from 158 subjects in 2 countries. Other studies have examined the subgingival microbiota of periodontally healthy subjects but were more limited in terms of numbers of samples and subjects. These studies examined a limited range of bacterial species by cultural (Dahlén et al. 1992, Moore & Moore 1994, Ali et al. 1997, Tanner et al. 1996, 1998, Macuch & Tanner 2000), darkfield (Offenbacher et al. 1985), antibody (Riviere et al. 1996, Di Murro et al. 1997) or molecular (Ali et al. 1997, Avila-Campos & Velasquez-Melendez 2002, Shiloah et al. 2000, Klein & Goncalves 2003) techniques. Many of the studies compared the microbiota of periodontal health with that of perio-

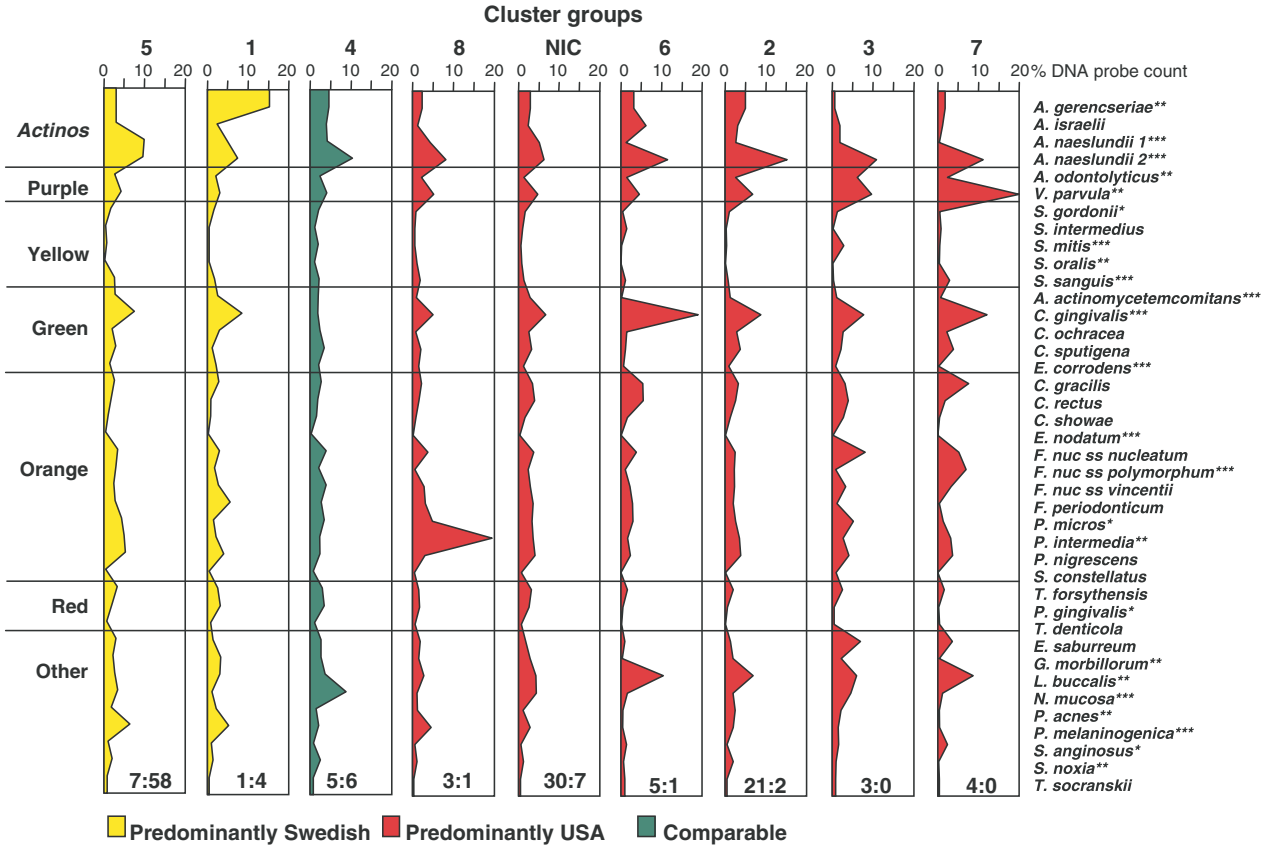

Fig. 4. Mean microbial profiles of the subjects in the 8 cluster groups depicted in Fig. 3 as well as the subjects who were ''not in cluster'' (NIC). Each panel represents the data from a single cluster or the NIC. The *x*-axis values indicate the mean proportions of each species in each cluster group. The color of each panel indicates whether the subjects in the cluster were predominantly from the USA or Sweden. The ratios at the bottom of each panel represent the number of American to Swedish subjects in each cluster group. Significance of differences of each species among cluster or NIC groups was determined using the Kruskal–Wallis test and adjusted for multiple comparisons; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

dontitis and or gingivitis and demonstrated relatively higher proportions/counts/prevalence of ''periodontal pathogens'' in samples from periodontally diseased subjects (Dahlén et al. 1992, Moore & Moore 1994, Riviere et al. 1996, Ali et al. 1997, Di Murro et al. 1997, Tanner et al. 1996, 1998, Macuch & Tanner 2000, Avila-Campos & Velasquez-Melendez 2002, Klein & Goncalves 2003). Ximenez-Fyvie et al. (2000) examined both the subgingival and supragingival microbiota of 22 periodontally healthy individuals using checkerboard DNA–DNA hybridization. Haffajee et al. (1998) compared the subgingival microbiota of periodontal health with that found in well-maintained, treated periodontitis subjects and subjectsd with chronic periodontitis. All of these studies indicated a shift in the microbiota from periodontal health to periodontal disease. However, these studies did not compare the microbiota of periodontal health or minimal periodontal disease in different geographic locations or examine the heterogeneity that may be found in the subgingival microbial profiles among such subjects.

The major finding of this study was that subgingival microbial profiles in periodontally healthy or minimally diseased subjects differed among subjects and between geographic locations. The reasons for these differences are not clear. Some of the dissimilarities in microbial profiles may be due to slight variation in periodontal status, although all subjects in the current investigation were selected using the same inclusion/exclusion criteria. The microbial differences might also be due to genetic background, diet, smoking status, socioeconomic status, racial/ethnic or cultural background of the subjects. The fact that the subjects in the current investigation have different subgingival microbiotas raises a number of questions. The key question of why such differences exist has already been discussed. A second question is what effect these differences in the subgingival microbiota in the periodontally healthy or minimally diseased state would have on the initiation and progression of periodontal diseases; are certain microbial profiles more likely to lead to periodontal stability, while others may lead to periodontal disease? Would certain microbial profiles lead to one form of periodontitis and others to a different form? Do microbial profiles of individuals with little or no disease shift from one pattern to another? Can a therapist alter subgingival microbial profiles in such individuals? The recognition of different subgingival microbial profiles in individuals prior to the onset of various forms of periodontitis may lead to strategies for the prevention of periodontal diseases.

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