Oral Bacterial Communities in Individuals with Type 2 Diabetes Who Live in Southern Thailand

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Type 2 diabetes mellitus is increasingly common in Thailand and elsewhere. In the present investigation, the bacteriological composition of saliva and supragingival plaque in Thai diabetics with and without active dental caries and in nondiabetics was determined by differential culture and eubacterial DNA profiling. Potential associations between fasting blood sugar and glycosylated hemoglobin (biomarkers of current and historical glucose control, respectively) with decayed, missing, and filled teeth and with salivary *Streptococcus* and *Lactobacillus* counts were also investigated. The incidence of active dental caries was greater in the Thai diabetics than in nondiabetics, and the numbers of total streptococci and lactobacilli were significantly higher in supragingival plaque from diabetics than in nondiabetics. *Lactobacillus* counts in the saliva and supragingival plaque of diabetics with active caries were significantly higher than those in diabetics without active caries. Oral eubacterial DNA profiles of diabetic versus nondiabetic individuals and of diabetics with active caries versus those without active caries could not be readily differentiated through cluster analysis or multidimensional scaling. The elevated caries incidence in the Thai diabetics was positively associated with numbers of bacteria of the acidogenic/acid-tolerant genera *Streptococcus* and *Lactobacillus*. *Lactobacillus* bacterial numbers were further elevated in diabetics with active caries, although salivary eubacterial DNA profiles were not significantly altered.

Diabetes mellitus (DM) represents a group of metabolic diseases characterized by hyperglycemia that result from defects in insulin secretion from the pancreas and/or insulin action (1). The three main types of diabetes are termed type 1, type 2, and gestational diabetes (2). The International Diabetes Federation estimated the worldwide incidence of diabetes among adults over 20 years of age to be 371 million in 2012, with India and China accounting for 92 million and 63 million cases, respectively, and Thailand accounting for 3.4 million (3). Type 2 diabetes is the most common form of the condition at ca. 95% of all cases.

Chronic hyperglycemia in diabetes is associated with long-term tissue damage, potentially leading to the dysfunction and failure of various organs (2, 4–7) and increasing the risk of oral diseases, including periodontal disease (8, 9), dental caries (10), and xerostomia (chronic dry mouth) (11). Xerostomia and hyposalivation reportedly occur in 12.5 and 45% of diabetics, respectively, but in only 5 and 2.5% of healthy persons (12). Factors underlying the potential association of dental caries with diabetes have received comparatively little research attention (13). Collin et al. (14) reported that the occurrence of dental caries did not differ between diabetics and healthy persons in Finland, while Hintao et al. (10) suggested that diabetes was a significant risk factor for root caries. While the reasons for the reported increased incidence of dental caries in diabetics remain unclear (13), it may be associated with higher numbers of oral pathogens such as *Streptococcus mutans* and lactobacilli in their saliva. In two independent studies, however, counts of *S. mutans* and lactobacilli in saliva were not significantly different in diabetic and nondiabetic individuals (14, 15). In terms of the bacteriological composition of plaque in diabetics, DNA hybridization has indicated an elevated abundance of *Treponema denticola*, *Prevotella nigrescens*, *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus intermedius* in the supragingival plaque of diabetics (15).

There are relatively few studies in the literature concerning the

bacterial profiles of saliva and supragingival plaque in diabetic patients, and to date, none have investigated potential associations between the bacterial profiles of saliva, supragingival plaque, and carious lesions obtained from diabetic patients in Thailand. The aims of this pilot study, therefore, were to conduct a cross-sectional study of potential differences between bacterial communities in diabetics and nondiabetics living in southern Thailand and to profile oral bacterial consortia at different sites in diabetic patients with and without active caries. Samples were analyzed by differential quantitative culture, combined with PCR-denaturing gradient gel electrophoresis (DGGE) (16), and dominant DGGE bands were sequenced for identity (17, 18). Potential associations between fasting blood sugar (FBS) and glycosylated hemoglobin (HbA1c) levels with numbers of salivary streptococci and lactobacilli were also assessed.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, the chemicals used throughout this study were of the highest grade available and were obtained from Sigma-Aldrich (Poole, United Kingdom). Formulated bacteriological media were obtained from Oxoid (Hampshire, United Kingdom). Bacteriological media were prepared according to the instructions provided by the manufacturer.

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Subject selection. This study was approved by the Ethics Committee of the Faculty of Medicine and the Faculty of Dentistry, Prince of Songkla University (PSU), Hat-Yai, Thailand. Informed consent was obtained before the study was performed. Twenty individuals with type 2 diabetes (15 females, 5 males) 42 to 78 years old (mean age, 56.4 years) and 11 nondiabetic volunteers (7 females, 4 males) 30 to 65 years old (mean age, 37.1 years) were investigated. The diabetic group was divided into two subgroups (10 individuals in each) according to the presence or absence of active caries. Patients who were diagnosed with other diseases, such as chronic pancreatitis, before a diabetes diagnosis and persons with other types of diabetes were excluded. The diagnosis and classification of diabetes followed the categories recommended by the American Diabetes Association (2). All diabetic patients were invited from the Endocrinological Clinic of the Songklanakarin Hospital Faculty of Medicine. Participants were selected by random sampling and then separated into three study groups, nondiabetic persons, diabetics without active caries, and diabetics with active caries. The nondiabetic group comprised metabolically healthy individuals. All diabetic patients and healthy controls were invited to have a dental examination. All diabetic patients' information files were reviewed until the most recent dental examination. The duration of disease, FBS level, concentration of HbA1c in blood serum (a biomarker of long-term glucose control), and treatment options (dietary control, oral medication, insulin injection) were obtained from hospital charts. Other information that related to a person's background and behavior (such as family history of DM, history of excessive consumption of sweet foods, history of smoking, and dry mouth) was recorded before the dental examination and treatment were completed. Caries prevalence was recorded according to the DMFT index, where D refers to the number of untreated carious teeth, F represents the number of filled teeth, M refers to the number of teeth extracted because of caries, and T refers to the total number of teeth (19–23). The DMFT index provides an indication of caries experience (current or previous caries activity). Teeth that were extracted because of periodontal disease or for orthodontic reasons were excluded from this index. HbA1c is a long-term biomarker of plasma glucose control that indicates how patients controlled their plasma glucose levels. Patients with poor glucose control had HbA1c levels of $\geq 7\%$ (24, 25).

Sampling. Unstimulated saliva was collected from all patients into sterile Universal bottles by expectoration over 5 min and carried from the clinic to the laboratory for serial dilution, which was completed within 30 min of sample collection. A portion of each inoculum was aliquoted and archived at -60°C for subsequent analysis.

Dental plaque samples were freshly obtained for all of the investigations. Pooled supragingival plaque from healthy persons was collected, and pooled plaque from persons with active caries was collected separately from two types of sites, the surface of healthy enamel and the surfaces of cavitated lesions. Plaque samples were collected separately with a sterile EXC 23 dental spoon excavator (Sci-Dent, Inc., Hamburg, NY), a sterile no. 40–41 dental spoon excavator (American Eagle Instruments Inc., Missoula, MT), or a sterile CD89/92 discoid-cleoid carver (Hu-Friedy Mfg. Co., Inc., Chicago, IL). These three instruments had the same blade size. Once collected, plaque samples were put into sterile Eppendorf tubes and weighed before use. For bacteriological analyses, plaque was transferred to 1 ml of half-strength thioglycolate broth for serial dilution. Samples also were archived at -60°C for subsequent analysis. Excavated carious (degraded) dentine from cavitated lesions was also collected after rinsing to remove debris. The sample sites were isolated and dried with a rubber dam sheet and an air spray, and carious dentine was removed with sterile dental spoon excavators as outlined above. As for dental plaque samples, carious dentine samples were processed immediately and also archived at -60°C for subsequent analyses.

Differential bacteriological analyses. For bacterial enumeration, samples of human saliva and dental plaque were homogenized by vortexing for 60 s. Samples were then serially diluted in sterile, prerduced, half-strength thioglycolate medium (USP). Appropriate dilutions (0.1

ml) were then plated in triplicate onto a variety of proprietary agar media to differentially isolate and enumerate various functional groups of oral bacteria: Wilkins Chalgren agar (incubated aerobically or in an anaerobic cabinet) for total aerobes and total anaerobes, respectively; Wilkins Chalgren agar with Gram-negative supplement; Trypticase yeast extract cysteine sucrose agar (26, 27) for streptococci and Rogosa agar for total lactobacilli. After plating, media were transferred immediately to an anaerobic cabinet (10:10:80 $\text{H}_2\text{-CO}_2\text{-N}_2$ gas mixture; Don Whitley Scientific, Shipley, United Kingdom) and incubated at $37 \pm 0.5^{\circ}\text{C}$, with the exception of the total aerobe count samples, which were incubated in a benchtop incubator (Cole-Parmer, London, United Kingdom) at 37°C for up to 5 days.

DNA extraction for eubacterium-specific PCR-DGGE. DNA was extracted from the archived saliva, dental plaque, and carious (degraded) dentine with QIAamp Mini Stool kits (Qiagen Ltd., West Sussex, United Kingdom) in accordance to the manufacturer's instructions. Samples were additionally processed by three alternating cycles of bead beating and incubation on ice (45 s each) or centrifuged for 1 min ($10,000 \times g$) as previously validated. The quality of extracted DNA was assessed by the electrophoresis of 5- μl aliquots on a 1% agarose gel containing GelRed DNA stain (from a 10,000 $\times$ stock solution; Biotium). All samples were run alongside a 1-kb DNA ladder (5 μl ; Bioline Ltd., London, United Kingdom). DNA extracts were stored at -60°C prior to analysis in nuclease-free containers.

PCR amplification for DGGE analysis. The V2–V3 region of the eubacterial 16S rRNA gene was amplified with eubacterium-specific primers HDA1–GC and HDA2 as previously described (28). PCRs were performed in sterile, nuclease-free, 0.2-ml tubes with a DNA thermal cycler (model 480; Perkin-Elmer, Cambridge, United Kingdom). In all cases, reactions were carried out with Red Taq DNA Polymerase Ready Mix (25 μl), HDA primers (2 μl of each, 5 μM), nanopure water (16 μl), and extracted community DNA (5 μl ; total DNA, ca. 10 ng). Amplification reactions were carried out with the following thermal program: 94°C (4 min), followed by 30 thermal cycles of 94°C (30 s), 56°C (30 s), and 68°C (60 s). The final cycle incorporated a 7-min chain elongation step (68°C). A D-Code Universal Mutation Detection System (Bio-Rad, Hemel Hempstead, United Kingdom) with 10% polyacrylamide gels (16 by 16 cm, 1 mm deep) run with 7 liters of $1\times$ TAE buffer solution diluted from $50\times$ TAE buffer (40 mM Tris base, 20 mM glacial acetic, 1 mM EDTA) was used to analyze community DNA amplicon mixtures. Denaturing gradients for parallel DGGE analysis ranged from 30 to 60%, as previously validated (29, 30). Gels were polymerized by adding tetramethylethylenediamine (50 μl) and ammonium persulphate (1% [wt/vol], 100 μl) immediately prior to pouring. Approximately 100 ng of purified PCR product was added in 5 μl of gel loading dye, and the total volume was made 10 μl with distilled water. This was then loaded onto gels, and electrophoresis was carried out at 90 V $\cdot$ h at 60°C for approximately 8 h. Gels were stained with SYBR gold stain (diluted 10^{-4} in $1\times$ TAE; Molecular Probes, Leiden, The Netherlands) for 30 min. Gels were visualized with a UV transilluminator at 312 nm (Peqlab Biotechnologies, Erlangen, Germany) and imaged with a Canon EOS 60 digital SLR system (Canon, Surrey, United Kingdom) with a SYBR photographic filter (Molecular Probes, Leiden, The Netherlands).

Partial 16S rRNA gene sequencing of excised bands. Bands representative of amplicons of interest were cut out of the gels with a sterile scalpel under UV transillumination, transferred to sterile, nuclease-free tubes together with 20 μl of nanopure water, and then incubated at 4°C for 20 h. Portions (5 μl) were removed and used as templates for reamplification. PCR products were purified with a QIAquick PCR purification kit (Qiagen Ltd., West Sussex, United Kingdom) and sequenced with the reverse primer HDA2 (non-GC clamp) at the University of Manchester DNA sequencing facility. The sequencing reaction protocol was as follows: 94°C (4 min), followed by 25 cycles of 96°C (30 s), 50°C (15 s), and 60°C (4 min). Once chain termination was complete, unidirectional DNA sequences were checked with CHROMAS-LITE (Technelysium Pty. Ltd., South Brisbane, Australia). The BLAST program

TABLE 1 Biological data for diabetes patients in this study

Variable	Diabetics		Nondiabetics (n = 11)
	Without active caries (n = 10)	With active caries (n = 10)	
Avg age in yr (range)	57.3 (42–78)	55.5 (43–73)	37.1 (30–65)
No. of females, males	6, 4	9, 1	7, 4
Mean duration of DM (yr) ± SD	7.20 ± 5.27	5.90 ± 2.96	NA ^a
FBS level (mg/dl) ± SD	154 ± 73.1	167 ± 54.3	NA
% with FBS level of >150 mg/dl (mean level [mg/dl] ± SD)	30 (239 ± 86.5)	60 (206 ± 38.9)	NA
% with poor glucose control ^b (mean HbA1c level [% wt/vol] ± SD)	40 (7.88 ± 0.87)	70 (8.66 ± 1.43)	NA
Mean plaque index ± SD	0.46 ± 0.54	0.96 ± 0.64	0.48 ± 0.6
Mean DMFT index ± SD	16.6 ± 9.22	16.4 ± 7.46	6.8 ± 0.46

^a NA, not applicable.^b HbA1c level of ≥7%.

(<http://www.ncbi.nlm.nih.gov/blast>) was used to search the European Molecular Biology Laboratories (EMBL) prokaryote database for sequences that matched.

Analysis of DNA profiles. All negative images of stained DGGE gels were separately aligned using Adobe Photoshop Elements 7.0 (Adobe, London, United Kingdom). Merged and aligned gel images were then analyzed with Bionumerics v.5.1 (Applied Maths, Sint-Martens-Latem, Belgium). In-software normalization generated a horizontally aligned gel image suitable for comparison. Accurate alignment of profiles was achieved by comparing replicated samples across gels and by confirming the identities of key band positions on the constructed gel image. Each lane on the gel was selected manually and then compared to the reference lane, allowing a matching profile for each lane to be generated by cluster analysis. In order to test for potential differences in oral (salivary) microbiota composition in diabetic and nondiabetic individuals and in diabetics with and without active caries, binary band matching profiles for each lane were analyzed with PRIMER software (v. 6) (Primer-E Ltd., Luton, United Kingdom) as follows. Bray-Curtis similarity values were calculated for imported data, and agglomerative hierarchical clustering was done via the CLUSTER menu of the PRIMER software. Similarity profile permutation tests were used to test for statistically significant evidence of genuine clusters, and data were further analyzed by using the nonmetric multidimensional scaling (MDS) algorithm. In order to test the significance of potential differences in consortial profiles, analysis of similarity (ANOSIM) was done with the ANOSIM test. DGGE data were also assessed by generating dendrograms through the unweighted-pair group method using average linkages with the Dice coefficient of similarity (16) and through principal-component analysis (PCA), as previously outlined by Humphreys and McBain (31).

Statistical analyses. Continuously variable results were presented by descriptive analysis as the sample sizes and means ± standard deviations, and their normality was checked with the Shapiro-Wilk test. Data were analyzed with the Statistical Package for Social Sciences (SPSS). The Student *t* test was used to compare the means of two groups. A forward linear regression was constructed with SPSS v.16.0 to examine two relationships.

The Shannon-Wiener index of diversity (H') was used to determine the diversity of eubacteria present in each sample with the following equation:

$$H' = -\sum_{i=1}^S (P_i) (\log_e P_i)$$

where s is the number of species (species richness) and P_i is the proportion of species in sample i . Shannon-Wiener indices were compared among saliva, supragingival plaque, and degraded dentine samples from diabetic patients with caries in each testing interval by using the Mann-Whitney *U* test performed with SPSS v.16.0.

RESULTS

Analysis of clinical characteristics of type 2 DM patients. There were no significant differences between diabetic and nondiabetic

patients with respect to age range and gender distribution. The diabetic group, however, had a DMFT score significantly higher than that of the healthy volunteers ($P < 0.05$). Biological data for diabetic volunteers with and without active caries are shown in Table 1, where DMFT indices of diabetes volunteers without caries were not statistically significantly different. However, plaque index scores were higher in the group of diabetics with active caries. Moreover, higher levels of the biological markers of glucose control, FBS and HbA1c, were detected in the serum of diabetics with active caries than in the serum of diabetics without active caries (Table 1), but the differences were not statistically significant.

Bacteriological composition of oral samples from healthy volunteers and diabetic patients. Viable counts of five major functional groups of oral bacteria, total facultative anaerobes, total anaerobes, total Gram-negative anaerobes, total streptococci, and total lactobacilli, in saliva, supragingival plaque, and carious (degraded) dentine obtained from diabetic and nondiabetic volunteers are shown in Fig. 1. The bacteriological compositions of materials from different samples sites (saliva, supragingival plaque, and degraded dentine) in diabetic volunteers are shown in Fig. 2. The mean numbers of total streptococci and lactobacilli were approximately 6 and 4 log₁₀ CFU/ml, respectively. The mean total counts of facultative anaerobes, anaerobes, and Gram-negative anaerobes ranged from ca. 8 to 9 log₁₀ CFU/ml (Fig. 2).

In terms of differences in the abundance of bacterial groups between the various test groups, salivary and plaque total counts of streptococci were significantly higher ($P < 0.05$) in diabetics than in nondiabetics and total numbers of lactobacilli were significantly higher in plaque from diabetics than in plaque from nondiabetics ($P < 0.05$). Figure 2 shows that the total numbers of streptococci in supragingival plaque obtained from diabetics with active caries were significantly higher than those in carious lesions ($P < 0.05$) while lactobacillus numbers in saliva and supragingival plaque from diabetics without active caries were significantly lower ($P < 0.05$) than in samples from those with active caries. Interestingly, in diabetics with active caries, *Lactobacillus* counts in degraded dentine were considerably higher than those in supragingival plaque samples.

With respect to the compositional similarity of samples, cluster analysis, MDS, and ANOSIM indicated that salivary eubacterial consortia did not differ significantly between diabetic and nondiabetic individuals (Fig. 3) and that significant differences could not be detected in the salivary eubacterial

FIG 1 Numbers of major groups of oral bacteria in diabetic and nondiabetic individuals. DS, diabetic saliva; NDS, nondiabetic saliva; NDP, nondiabetic dental plaque; DP, diabetic dental plaque; DC, diabetic caries/degraded dentine. The upper and lower lines in the box plots indicate the first and third quartiles, the central horizontal line represents the median, and the whiskers indicate the minimum and maximum values. Significance ($P < 0.05$) is indicated by asterisks. $n = 20$ (10 for DC). Outlying values have been omitted for clarity.

DNA profiles of diabetics with active caries with and those without active caries (Fig. 4). These observations were supported by PCA (data not shown).

Figure 3 shows a hierarchical clustering plot of salivary eubacterial communities from diabetics and nondiabetics. Significant clusters were not detected, and likewise, samples were not differentiated on the basis of diabetes in the two-dimensional MDS plot (Fig. 3b), an observation that is supported by the relatively high stress value (0.264). This was additionally corroborated by ANOSIM, where $R = -0.009$ and $P = 0.525$. Cluster analysis and MDS of salivary eubacterial communities from diabetics with and

without active caries are shown in Fig. 4a and b, respectively. No obvious differentiation was apparent, this time, on the basis of caries status. $R = 0.076$ and $P = 0.139$ by ANOSIM.

Analysis of DGGE fingerprints, where the number of bands is broadly proportional to eubacterial diversity, enables bacterial diversity to be compared between samples. Shannon-Wiener values for salivary DGGE profiles did not indicate an association between eubacterial diversity and the sample site, disease status, or the presence of active caries (Table 2).

Sequence analysis of selected DGGE amplicons. Selected DGGE bands were excised and sequenced for identity following

FIG 2 Numbers of major groups of oral bacteria in saliva and dental plaque of diabetics with and without active dental caries. NCS, noncaries saliva; CS, caries saliva; NCP, noncaries dental plaque; CP, carious dental plaque; CL, carious lesions/degraded dentine. The upper and lower lines in the box plots indicate the first and third quartiles; the central horizontal line represents the median, and the whiskers indicate the minimum and maximum values. Significance ($P < 0.05$) is indicated by asterisks. $n = 10$. Outlying values have been omitted for clarity.

identification by using a synthetic reference lane that was generated with BioNumerics software. For each band in the synthetic reference lane, corresponding excised bands were identified for subsequent sequence analysis. Table 3 indicates the closest taxonomic relatives of sequenced bands, based on results of BLAST searches. In all cases, bands in the same position but in different lanes were excised and sequenced to confirm that they had the same identity (data not shown). Data in Table 3 also include the incidence of each bacterium in diabetics with and without active caries and also show the bacterial diversity at different sites within the mouths of diabetes patients.

According to sequence analysis, compared to the predominant species in the saliva of diabetics without active caries, bacteria with homology to *Lactobacillus fermentum*, *Streptococcus* sp., and *Actinomyces viscosus* occurred at a lower incidence; while species including *Capnocytophaga* sp., *Prevotella multisaccharivorax*, *Streptococcus mutans*, *Lautropia* sp., *Veillonella parvula*, *Neisseria mucosa*, *Selenomonas* sp., and *Rothia dentocariosa* dominated. The microbiota of the dental plaque overlying carious lesions was characterized by its comparatively lower microbial diversity in diabetes patients than in similar plaque overlying healthy teeth. *Bacteroides vulgatus* was detected with the greatest frequency

FIG 3 Salivary DGGE profiles of diabetic and nondiabetic individuals analyzed by cluster analysis (a) and nonmetric MDS (b). Open symbols, nondiabetics; closed symbols, diabetics. Contour lines on the MDS plot superimpose 50% resemblance levels derived from cluster analysis. Caries-active and non-active individuals could not be differentiated on this basis. The lack of significant difference between groups was confirmed with the ANOSIM test in the PRIMER v6 software. $R = -0.009$ and $P = 0.525$.

(70%) in dental plaque overlying carious lesions from diabetes patients with active caries. An uncultured bacterium was the predominant organism in degraded dentine (frequency, 50%), while bacteria with homology to *Lactobacillus fermentum*, *Streptococcus mutans*, and *Propionibacterium propionicum* were the second most prevalent (frequency, 40%). Bacteria with homology to *Leuconostoc mesenteroides*, *Capnocytophaga gingivalis*, *Prevotella multisaccharivorax*, *Capnocytophaga* sp., *Neisseria subflava*, and *Corynebacterium matruchotii* occurred with greater frequency in degraded dentine than in dental plaque overlying caries. *Streptococcus mutans*, *Lactobacillus fermentum*, and *Actinomyces viscosus* occurred in all samples from diabetes patients with and without active car-

FIG 4 Salivary DGGE profiles of diabetics with and without active dental caries analyzed by cluster analysis (a) and nonmetric MDS (b). Open symbols, nondiabetics; closed symbols, diabetics. Contour lines on the MDS plot superimpose 50% resemblance levels derived from cluster analysis. Diabetic and nondiabetic individuals could not be differentiated on this basis. The lack of significant difference between groups was confirmed with the ANOSIM test in the PRIMER v6 software. $R = 0.076$ and $P = 0.139$.

ies. In contrast, *Streptococcus sanguinis* was found only in saliva obtained from diabetes patients without active caries (frequency, 10%) and in dental plaque overlying noncarious teeth obtained from diabetics with active caries (frequency, 50%).

TABLE 2 Shannon-Wiener indices of bacterial communities from different sample sites derived from diabetes patients with caries and from the saliva of nondiabetics

Group and sample	Mean Shannon-Wiener index (SD) ^a
Diabetics	
Saliva	2.92 (0.27)
Dental plaque from noncarious teeth	3.08 (0.23)
Dental plaque from teeth with active caries	3.04 (0.54)
Degraded dentine	2.81 (0.39)
Nondiabetics, saliva	3.02 (0.27)

^a The differences between groups are not statically significant ($P > 0.05$).

TABLE 3 Sequences of PCR amplicons derived from DGGE and identities based on the BLAST database derived from diabetes patients with and without caries

Sequence no.	Closest relative, accession no. (% sequence similarity) ^a	Sequence length (bp) ^b	No. of volunteers (% incidence) ^c					
			DMNC-S	DMC-S	DMNC-P	DMC-PNC	DMC-PC	DM-C
1	<i>Capnocytophaga gingivalis</i> , GU410488 (100)	193 (0)	2 (20)	0 (0)	1 (10)	4 (40)	4 (40)	2 (20)
2	<i>Leuconostoc mesenteroides</i> , GU907675 (98)	173 (0)	1 (10)	3 (30)	1 (10)	2 (20)	0 (0)	2 (20)
3	<i>Capnocytophaga granulosa</i> , GU410101 (92)	150 (1)	3 (30)	3 (30)	2 (20)	3 (30)	5 (50)	3 (30)
4	<i>Capnocytophaga</i> sp. oral taxon, GU412131 (100)	158 (0)	7 (70)	6 (60)	1 (10)	2 (20)	6 (60)	3 (30)
5	<i>Leuconostoc citreum</i> , FN796873 (97)	168 (2)	1 (10)	4 (40)	3 (30)	2 (20)	3 (30)	4 (40)
6	<i>Lactobacillus fermentum</i> , GU417381 (98)	169 (0)	1 (10)	3 (30)	3 (30)	5 (50)	4 (40)	4 (40)
7	<i>Capnocytophaga gingivalis</i> , GU410378 (95.5)	281 (1)	3 (30)	4 (40)	2 (20)	4 (40)	6 (60)	3 (30)
8	<i>Staphylococcus aureus</i> , GU594473 (99)	172 (0)	6 (60)	6 (60)	6 (60)	1 (10)	3 (30)	2 (20)
9	<i>Pseudomonas</i> sp., EF517951 (95)	161 (1)	6 (60)	7 (70)	5 (50)	8 (80)	4 (40)	3 (30)
10	<i>Fusobacterium nucleatum</i> subsp. <i>polymorphum</i> , AB550231 (91)	262 (4)	4 (40)	4 (40)	5 (50)	3 (30)	4 (40)	1 (10)
11	<i>Capnocytophaga ochracea</i> , GU561330 (98)	169 (4)	3 (30)	1 (10)	4 (40)	3 (30)	2 (20)	0 (0)
12	NS	150 (2)	1 (10)	1 (10)	4 (40)	4 (40)	3 (30)	3 (30)
13	<i>Capnocytophaga gingivalis</i> , GU410488 (97)	150 (1)	3 (30)	3 (30)	3 (30)	4 (40)	3 (30)	4 (40)
14	<i>Prevotella multisaccharivorax</i> , GU427588 (100)	148 (0)	6 (60)	4 (40)	5 (50)	6 (60)	4 (40)	4 (40)
15	<i>Bacteroides vulgatus</i> , CP000139 (78)*	141 (0)	2 (20)	2 (20)	2 (20)	3 (30)	7 (70)	3 (30)
16	NS	149 (0)	2 (20)	4 (40)	2 (20)	4 (40)	3 (30)	3 (30)
17	<i>Prevotella multisaccharivorax</i> , AB200414 (98)	150 (0)	2 (20)	2 (20)	2 (20)	3 (30)	1 (10)	2 (20)
18	<i>Streptococcus mutans</i> , GU424617 (100)	174 (1)	6 (60)	4 (40)	6 (60)	3 (30)	5 (50)	4 (40)
19	<i>Lautropia</i> sp. oral taxon, GU428984 (97)	170 (3)	7 (70)	4 (40)	4 (40)	4 (40)	2 (20)	2 (20)
20	<i>Corynebacterium</i> sp. oral taxon, GU429038 (100)	161 (0)	3 (30)	3 (30)	2 (20)	3 (30)	3 (30)	1 (10)
21	Uncultured bacterium, GQ034914 (94)	159 (1)	0 (0)	2 (20)	1 (10)	1 (10)	2 (20)	0 (0)
22	<i>Neisseria</i> sp., EM889136 (78.9)*	147 (0)	0 (0)	2 (20)	0 (0)	3 (30)	0 (0)	0 (0)
23	<i>Streptococcus sanguinis</i> , GU426625 (93)	154 (1)	1 (10)	0 (0)	0 (0)	5 (50)	0 (0)	0 (0)
24	<i>Capnocytophaga</i> sp. oral taxon, GU470889 (94)	143 (0)	4 (40)	0 (0)	2 (20)	5 (50)	1 (10)	2 (20)
25	Uncultured bacterium, FJ535449 (92)	145 (1)	2 (20)	2 (20)	1 (10)	1 (10)	1 (10)	2 (20)
26	<i>Streptococcus</i> sp. oral taxon, GU432139 (95)	149 (0)	0 (0)	2 (20)	1 (10)	1 (10)	1 (10)	0 (0)
27	<i>Capnocytophaga</i> sp., GU410274 (97)	165 (3)	1 (10)	2 (20)	3 (30)	2 (20)	3 (30)	3 (30)
28	Uncultured bacterium, HM323408 (98)	158 (2)	0 (0)	2 (20)	0 (0)	4 (40)	2 (20)	5 (50)
29	<i>Veillonella parvula</i> , GU406864 (94)	157 (0)	5 (50)	3 (30)	3 (30)	1 (10)	3 (30)	0 (0)
30	<i>Veillonella parvula</i> , CP001820 (78.8)*	150 (3)	4 (40)	3 (30)	2 (20)	0 (0)	4 (40)	0 (0)
31	<i>Prevotella tannerae</i> , GU413107 (94)	153 (1)	1 (10)	3 (30)	4 (40)	1 (10)	1 (10)	0 (0)
32	<i>Corynebacterium matruchotii</i> , GU420818 (99)	165 (2)	1 (10)	2 (20)	2 (20)	0 (0)	0 (0)	0 (0)
33	<i>Neisseria mucosa</i> , GU423117 (99)	169 (2)	4 (40)	0 (0)	2 (20)	1 (10)	1 (10)	1 (10)
34	<i>Neisseria subflava</i> , GU413333 (90)	165 (0)	1 (10)	3 (30)	2 (20)	0 (0)	0 (0)	1 (10)
35	<i>Prevotella oris</i> , GU409760 (97)	150 (1)	1 (10)	1 (10)	1 (10)	3 (30)	3 (30)	1 (10)
36	Uncultured bacterium, HM345119 (100)	159 (0)	2 (20)	1 (10)	1 (10)	1 (10)	1 (10)	1 (10)
37	<i>Prevotella tannerae</i> , GU413080 (95)	147 (0)	0 (0)	0 (0)	2 (20)	1 (10)	2 (20)	0 (0)
38	<i>Corynebacterium matruchotii</i> , GU420869 (91)	147 (1)	0 (0)	1 (10)	1 (10)	1 (10)	1 (10)	1 (10)
39	<i>Prevotella nigrescens</i> , GU424682 (97)	159 (0)	0 (0)	2 (20)	1 (10)	2 (20)	2 (20)	1 (10)
40	<i>Corynebacterium matruchotii</i> , GU420729 (100)	164 (1)	0 (0)	0 (0)	1 (10)	2 (20)	2 (20)	1 (10)
41	<i>Propionibacterium propionicum</i> , GU425701 (99)	134 (0)	2 (20)	1 (10)	0 (0)	1 (10)	2 (20)	1 (10)
42	<i>Propionibacterium propionicum</i> , GU425679 (96)	263 (1)	4 (40)	5 (50)	5 (50)	6 (60)	4 (40)	4 (40)
43	<i>Selenomonas</i> sp. oral taxon, GU431032 (91)	164 (0)	5 (50)	2 (20)	0 (0)	3 (30)	3 (30)	1 (10)
44	<i>Corynebacterium matruchotii</i> , GU420812 (96)	157 (0)	1 (10)	2 (20)	3 (30)	1 (10)	3 (30)	1 (10)
45	Uncultured bacterium, HM345119 (100)	167 (2)	0 (0)	2 (20)	0 (0)	1 (10)	0 (0)	1 (10)
46	<i>Corynebacterium</i> sp. oral taxon, GU429064 (99)	160 (0)	1 (10)	1 (10)	0 (0)	2 (20)	1 (10)	0 (0)
47	<i>Neisseria flavescens</i> , GU417565 (96)	157 (0)	0 (0)	0 (0)	0 (0)	1 (10)	2 (20)	2 (20)
48	<i>Corynebacterium</i> sp., AM420211 (100)	295 (4)	3 (30)	1 (10)	5 (50)	2 (20)	3 (30)	1 (10)
49	<i>Corynebacterium matruchotii</i> , GU420728 (100)	171 (2)	2 (20)	4 (40)	4 (40)	4 (40)	3 (30)	4 (40)
50	<i>Lautropia mirabilis</i> , GU397907 (100)	164 (0)	3 (30)	3 (30)	5 (50)	3 (30)	3 (30)	2 (20)
51	<i>Rothia dentocariosa</i> , GU416838 (100)	151 (0)	3 (30)	0 (0)	7 (70)	3 (30)	3 (30)	2 (20)
52	<i>Actinomyces viscosus</i> , GU561308 (99)	165 (0)	2 (20)	3 (30)	2 (20)	2 (20)	3 (30)	3 (30)

^a Similarities are based on pairwise alignments with published sequences according to BLAST searches and indicate similarity, not guaranteed identity. Sequence numbers 1 to 52 refer to consecutive DGGE band positions from the top to the bottom of the synthetic reference ladder. *, low homology to published sequences.

^b The number of ambiguous bases for an individual band is shown in parentheses.

^c Refers to the percent incidence of each unique band in diabetic individuals with and without caries. DMNC-S, saliva from diabetics without caries; DMC-S, saliva from diabetics with caries; DMNC-P, plaque from diabetics without caries; DMC-PNC, plaque from diabetics with caries on teeth with no caries; DMC-PC, plaque from diabetics with caries on teeth with caries; DM-C, degraded dentine from diabetics.

Potential correlations between putative biological markers and selected oral bacterial levels. Elevated FBS and HbA1c levels were associated with higher numbers of salivary streptococci (data not shown). Positive correlations were detected between FBS concentrations and salivary streptococci ($R^2 = 0.43$) and between

HbA1c levels and salivary streptococci ($R^2 = 0.42$). However, these relationships were not statistically significant ($P > 0.05$). There was also a positive correlation between FBS levels and salivary lactobacilli ($R^2 = 0.17$) and a moderately positive correlation between HbA1c levels and salivary lactobacilli ($R^2 = 0.32$). How-

ever, the relationships between FBS levels and salivary lactobacilli and between HbA1c levels and numbers of salivary lactobacilli were not statistically significant ($P > 0.05$). Moderate negative correlations between FBS and DMFT ($R^2 = -0.32$) and HbA1c and DMFT ($R^2 = -0.38$) were observed, but these were not statistically significant ($P > 0.05$) (data not shown).

DISCUSSION

The present investigation provides information regarding the relationship between diabetes and oral disease and the potential correlation between bacterial numbers and active caries in Thai individuals with diabetes. The mean age and gender distributions within the diabetic and nondiabetic groups were not significantly different and were broadly comparable to the reported global diabetes prevalence (32), and the greater incidence of active dental caries in diabetes patients than in nondiabetics was broadly in agreement with previous reports (10, 33).

With respect to volunteer numbers, a power calculation with data generated in the present study indicated that to detect a minimum of a 1.0-log change in *Lactobacillus* numbers would require between 8 and 22 volunteers (based on variations in plaque and salivary *Lactobacillus* counts, respectively) to achieve 90% statistical power at a two-sided 5% significance level. However, increasing numbers of volunteers would increase the statistical power of the study such that 43 volunteers would enable a 0.5-log increase in *Lactobacillus* numbers to be detected by using the same criteria.

The bacterial diversities in human saliva and supragingival dental plaque of diabetic and nondiabetic individuals were compared by combining PCR-DGGE of 16S rRNA genes with image analysis and sequencing of key PCR amplicons. This approach has been previously used to profile the composition of oral microbial communities (17, 34, 35). DGGE analysis has been previously used in a limited number of studies to produce fingerprints of saliva (36, 37) and supragingival plaque diversity (38, 39), and some of these studies have combined DGGE, cluster analysis, and sequencing. An advantage of this approach is that the detection threshold may be lower than for isolation techniques, particularly where ca. 50% of the target bacterial community may not be culturable (40). Also, the identity of any amplifiable target sequence may be gleaned even if it comprises a limited proportion of the total microbial population (41). Most of the clustering between individuals occurred at the 60 to 80% concordance, which is comparable to previous studies that have used this technique to compare salivary and other oral microbial samples (37, 38, 42–44) and also reflective of the level of interindividual variation observed in other microbiotas, including the gut (45–47).

While a number of putative clusters were apparent following the analysis of DGGE profiles, these did not relate to patient or disease groups. The numbers of salivary streptococci were significantly higher in diabetes patients than in healthy individuals in the present investigation. This contrasts with a previous study (15) where no significant difference between these two groups was observed. The frequent occurrence of *Streptococcus sanguinis*, *Actinomyces viscosus*, *Neisseria subflava*, and *Lautropia* sp. in supragingival plaque in the Thai subjects is broadly in agreement with a previous Malaysian study which similarly reported the presence of *Streptococcus oralis*, *Streptococcus sanguinis*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Lautropia* sp., *Kingella oralis*, *Neisseria subflava*, *Neisseria mucosa*, and *Rothia mucilaginosa* on the tooth surfaces of healthy persons (34). Hintao and colleagues reported

higher incidences of *Treponema denticola*, *Prevotella nigrescens*, *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus intermedius* in the supragingival plaque of diabetes patients than in that of healthy persons by the checkerboard DNA-DNA hybridization method (15), while the present study indicated elevated frequencies of *Prevotella nigrescens*, *Streptococcus sanguinis*, *Lactobacillus fermentum*, *Streptococcus mutans*, and *Actinomyces viscosus*.

In the present study, *Neisseria flavescens* was detected only in dental plaque and not in the saliva of either nondiabetic or diabetic individuals. *Neisseria*, an obligate aerobe, has been implicated in the early stages of dental plaque development (48). However, *Corynebacterium matruchotii* was found in both health and disease, an observation that contrasts with a previous study conducted in the United Kingdom (35) that found this bacterium to be unique to healthy persons. However, as well as the different geographical location, the United Kingdom study used samples of subgingival plaque exclusively, as opposed to the supragingival plaque used in the present investigation (35).

Data presented here show similar total salivary bacterial compositions in diabetics with and without active caries, according to DNA profiling. Furthermore, exhaustive sequencing of DGGE bands revealed no significant associations among the incidences of cariogenic bacteria in saliva, dental plaque, and degraded dentine in diabetics with active caries. This contrasts with a previous study that reported a strong association between cariogenic bacteria in saliva and both root surface and coronal caries. Chhour et al. (49) reported that lactobacilli and prevotellae were highly abundant in degraded dentine as determined by real-time PCR, in contrast to the present study, where the prevalence of lactobacilli in saliva, dental plaque, and degraded dentine was comparatively low, indicating the potential influence of volunteer selection and experimental design on the outcome obtained (15).

With respect to associations between past and current glucose control and numbers of streptococci and lactobacilli and caries experience, although the positive association between FBS/HbA1c and salivary streptococci/lactobacilli was not statistically significant, comparable associations have been previously reported (50). An important influence on the numbers of these important acidogenic and acid-tolerant (and thus potentially cariogenic) oral genera in individuals with type 2 diabetes is the salivary concentration of glucose. Accordingly, differences in salivary glucose concentration between diabetic and nondiabetic volunteers have been reported, with markedly higher concentrations occurring in diabetics than in nondiabetics (51). Since serum FBS/HbA1c levels are biomarkers of poor glucose control in diabetics (short and longer term, respectively); individuals with elevated levels of these biomarkers are likely to have had elevated salivary glucose concentrations for various amounts of time in the past.

Our data indicate that diabetes patients with good glucose control had a better past dental caries experience than did patients with poor control. This contrasts with the observations of Miralles et al. (33), who reported that blood glucose control (measured as HbA1c) was not related to the incidence of dental caries. This apparent difference could be due to the fact that the age range of volunteers in the present study was higher (42 to 78 years) than that in the previous study (18 to 50 years). Data in the present investigation also indicate that plaque indices were significantly higher in diabetes patients with active caries than in diabetes patients without active caries. Although the M (missing-tooth)

scores of diabetes patients without caries was lower than those of diabetes patients with caries, the F (filled-tooth) scores of diabetes patients without caries was higher than those of diabetes patients with caries. There was, however, no significant difference in dental caries experience between diabetes patients with and without active caries. While apparently counterintuitive, the negative association between FBS/HbA1c and DMFT may be explained by the fact that DMFT scores combine factors that may reflect current or recent cariogenic states (i.e., D [decay]) with past cariogenesis (i.e., F [filled teeth] and M [missing teeth]). On this basis, the correlation between HbA1c/FBS and D may be more likely to correlate positively than DMFT values since these reflect more-current events, which may be linked to elevated glucose biomarkers.

In summary, the elevated caries incidence in Thai diabetics was associated with elevated numbers of culturable streptococci and lactobacilli, which are acidogenic/acid-tolerant genera and thus potentially cariogenic. *Lactobacillus* numbers were further elevated in diabetics with active caries, although salivary eubacterial DNA profiles did not differ significantly in these individuals. The positive correlation between serum FBS/HbA1c levels and elevated numbers of streptococci and lactobacilli emphasizes the importance of effective glucose control for dental, as well as general, health in diabetes.

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