The bacterial microbiota in the oral mucosa of rural Amerindians

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The oral microbiota plays an important role in buccal health and in diseases such as periodontitis and meningitis. The study of the human oral bacteria has so far focused on subjects from Western societies, while little is known about subjects from isolated communities. This work determined the composition of the oral mucosa microbiota from six Amazon Amerindians, and tested a sample preservation alternative to freezing. Paired oral swabs were taken from six adults of Guahibo ethnicity living in the community of Platanillal, Amazonas State, Venezuela. Replicate swabs were preserved in liquid nitrogen and in Aware Messenger fluid (Calypte). Buccal DNA was extracted, and the V2 region of the 16S rRNA gene was amplified and pyrosequenced. A total of 17 214 oral bacterial sequences were obtained from the six subjects; these were binned into 1034 OTUs from 10 phyla, 30 families and 51 genera. The oral mucosa was highly dominated by four phyla: *Firmicutes* (mostly the genera *Streptococcus* and *Veillonella*), *Proteobacteria* (mostly *Neisseria*), *Bacteroidetes* (*Prevotella*) and *Actinobacteria* (*Micrococcineae*). Although the microbiota were similar at the phylum level, the Amerindians shared only 62 % of the families and 23 % of the genera with non-Amerindians from previous studies, and had a lower richness of genera (51 vs 177 reported in non-Amerindians). The Amerindians carried unidentified members of the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* and their microbiota included soil bacteria Gp1 (*Acidobacteriaceae*) and *Xylanibacter* (*Prevotellaceae*), and the rare genus *Phocoenobacter* (*Pasteurellaceae*). Preserving buccal swabs in the Aware Messenger oral fluid collection device substantially altered the bacterial composition in comparison to freezing, and therefore this method cannot be used to preserve samples for the study of microbial communities.

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INTRODUCTION

The oral cavity is a complex habitat for bacteria in healthy subjects, although bacteria also play a causative role in periodontal diseases (Kroes *et al.*, 1999). More than 600 different bacterial species have been described from the human oral cavity (Keijser *et al.*, 2008; Nasidze *et al.*, 2009a), but these studies have been limited to Caucasians

from modern societies in developed countries. Modern lifestyles involve the use of medicines (including antibiotics), and specific dietary and oral hygiene habits, gum chewing, amalgam fillings, smoking, and other factors that might affect the oral microbiota. The full diversity of the human microbiota can therefore be assessed only by studies in diverse human populations.

Recent improvements in DNA sequencing technology (e.g. barcoded pyrosequencing on the Genome Sequencer FLX/454 Life Sciences platform) have enabled a dramatic increase in throughput via parallel in-depth analysis of

Abbreviation: OTU, operational taxonomic unit.

Three supplementary tables are available with the online version of this paper.

many samples simultaneously (Hamady *et al.*, 2008; Meyer *et al.*, 2008). These techniques have been successfully applied to determine bacterial diversity in different human body habitats, including the oral cavity, intestine, vagina and skin (Bik *et al.*, 2006; Fierer *et al.*, 2008; Keijser *et al.*, 2008; Grice *et al.*, 2009; Nasidze *et al.*, 2009a; Zaura *et al.*, 2009; Domínguez-Bello *et al.*, 2010; Ravel *et al.*, 2010). In this work we describe the composition of the bacterial communities of the oral mucosa in South American Amerindians living in a remote village, less exposed to selective pressures of modern life, and also tested a chemical sample preservation method that provides an alternative to freezing, which might facilitate human microbiome studies in remote geographical locations.

METHODS

Subjects and samples. Six apparently healthy Amerindian adults (18 to 45 years old, five females and one male) of Guahibo ethnicity were sampled in the community of Platanillal, Amazonas, Venezuela, in June 2008 (Table 1). Volunteers signed an informed consent and the protocol was approved by the IVIC Bioethical Committee. Use of samples without identifiers was approved by the IRB at UPR. The village lacks electricity and running water, toilets are latrines and the people practise personal hygiene at the river or with buckets of water transported from the river or from one of the village wells. We did not study the oral health of the subjects, but most adults had evidence of dental cavities.

Two oral swab samples were taken from each individual, rubbing the cheek oral mucosa with a swab, five to seven times on each side. The swabs were cut and the tips were immediately preserved either in cryovials immersed in liquid nitrogen, or, in order to test the efficacy of a preserving solution, in an Aware Messenger oral fluid collection device (Calypse Biomedical Corporation) at room temperature. The Aware Messenger oral fluid collection device is marketed for the collection, stabilization and transport of oral fluid specimens. Aware has been tested to preserve IgG-rich oral fluid (<http://www.calypse.com/aware-messenger.asp>), but has not been tested for preservation of nucleic acids. After 3 weeks, when samples arrived at the laboratory, all swabs were kept in an ultralow-temperature freezer at -80°C until DNA extraction and analysis. We decided to test the Aware in the samples we were collecting from Amazonian peoples rather than in laboratory studies that would have required extra sequencing.

Table 1. Description of the Amerindian subjects included in this work

All the subjects were of Guahibo ethnicity.

Amerindian subject	Sex	Village	Age (years)
PA08-71	F	San Pablito	19
PA08-72	M	Platanillal	18
PA08-75	F	Platanillal	28
PA08-77	F	Platanillal	19
PA08-83	F	Platanillal	35
PA08-88*	F	Platanillal	45

*Taking amoxicillin at the time of sampling

DNA extraction. DNA was extracted from the oral swabs using the MO BIO Power Soil DNA Isolation kit after the swabs were put in bead tubes with 60 μl Solution C1. The tubes were incubated at 65°C for 10 min and vortexed for 2 min, followed by the procedures indicated by the manufacturer. Extracted DNA was stored at -20°C .

PCR amplification of the V2 region of bacterial 16S rRNA genes. The 16S rRNA gene was amplified using a primer set as described by Fierer *et al.* (2008). The forward primer (5'-GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AG-3') contained the 454 Life Sciences primer B sequence, the broadly conserved bacterial primer 27F, and a two-base linker sequence ('TC'). The reverse primer (5'-GCC TCC CTC GCG CCA TCA GNN NNN NNN NNN NCA TGC TGC CTC CCG TAG GAG T-3') contained the 454 Life Sciences primer A sequence, a unique 12 nt error-correcting Golay barcode used to tag each PCR product (designated by NNNNNNNNNNNN), the broad-range bacterial primer 338R, and a 'CA' linker sequence inserted between the barcode and the rRNA primer. PCRs were carried out in triplicate 25 μl reactions with 0.6 μM forward and reverse primers, 3 μl template DNA, and $1 \times$ HotMasterMix (5 PRIME). All PCRs were prepared using certified DNA-free PCR water (MO BIO) in a PCR hood UV irradiated for at least 30 min before use. Thermal cycling consisted of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s, with a final extension of 10 min at 72°C . Amplicons were cleaned using the UltraClean-htp 96-well PCR Clean-up kit (MO BIO) and DNA concentrations were measured using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen). Following quantification, cleaned amplicons were combined in equimolar ratios into a single tube, and DNA was precipitated on ice for 45 min after the addition of 5 M NaCl (0.2 M final concentration) and then 2 vols ice-cold 100% ethanol. The precipitated DNA was centrifuged at 7800 g for 40 min at 4°C , and the resulting pellet was washed with an equal volume of ice-cold 70% ethanol and centrifuged again at 7800 g for 20 min at 4°C . The supernatant was removed and the pellet was air-dried for 10 min at room temperature and resuspended in nuclease-free water (MO BIO).

Pyrosequencing and sequence analysis. Pyrosequencing was carried out on a 454 Life Sciences Genome Sequencer FLX instrument (Roche) by the Environmental Genomics Core Facility at the University of South Carolina (Columbia). Sequences (200–250 nt) were processed and analysed as described by Hamady *et al.* (2008), and assigned to operational taxonomic units (OTUs) using cd-hit (Li & Godzik, 2006) with an identity cut off of 97%.

Representative sequences were aligned using NAST (DeSantis *et al.*, 2006a) and the Greengenes database (DeSantis *et al.*, 2006b) with a minimum alignment length of 150 and a minimum identity of 75%. A phylogenetic tree was inferred using Clearcut (Sheneman *et al.*, 2006) with Kimura's two-parameter model. Taxonomy was assigned using the RDP classifier with a minimum support threshold of 60% (Wang *et al.*, 2007) and the RDP taxonomic nomenclature.

Community comparisons. To determine the amount of bacterial diversity shared between two communities (beta diversity) we employed the UniFrac metric (Lozupone & Knight, 2005; Lozupone *et al.*, 2007). UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from the 16S rRNA gene sequences from all communities being compared. A relatively small UniFrac distance implies that two communities are compositionally similar, containing lineages sharing a common evolutionary history. In unweighted UniFrac, only the presence or absence of lineages is considered. In weighted UniFrac, branch lengths are weighted based on the relative abundances of lineages within communities.

Fig. 1. Major bacterial phyla in the oral mucosa from six adult Guahibo Amerindians.

RESULTS

Oral bacterial diversity

A total of 17 162 oral bacterial sequences were obtained, 8696 from the frozen swabs and 8466 from the Aware-preserved swabs. Unclassified sequences accounted for 0.3 % of the total sequences. In total 1034 OTUs from 10 phyla were found in the oral mucosa community from samples preserved frozen or in Aware Messenger solution, although alpha diversity figures should be treated with caution as no denoising was performed on these samples. We found 693 OTUs from 10 phyla, 30 families and 51 genera, in the frozen oral mucosa swabs from the six Amerindians, 68.5 % of which were unique (i.e. found in one person). Each Amerindian oral frozen sample had on average 220 ± 39 OTUs.

The bacterial sequences from the oral mucosa were highly dominated by four phyla (Fig. 1; see also Supplementary Table S1, available with the online version of this paper): *Firmicutes* (mostly the genera *Streptococcus* and *Veillonella*), *Proteobacteria* (mostly *Neisseria*), *Bacteroidetes* (*Prevotella*) and *Actinobacteria* (*Micrococcineae*). Other phyla included *Fusobacteria*, SR1, TM7 and other (*Spirochaetes*, *Acidobacteria*, *Cyanobacteria*, *Tenericutes*). The Amerindians carried unidentified members of the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Supplementary Table S1). No evidence of a substantial difference in the bacterial community between the subject taking amoxicillin (Table 1) and the other subjects was found.

Effect of the alternative chemical sample preservation

Preserving buccal swabs in the Aware Messenger oral fluid collection device altered the bacterial community composition relative to ultralow-temperature freezing (Table 2, Supplementary Table S2). UniFrac-based principal coordinates analysis indicated that sample storage in Aware Messenger solution shifted the bacterial community structure, with PC1 separating samples according to preservation method and explaining as much as 56.4% of the total sample variation (Fig. 2). Relative to frozen samples, Aware-preserved samples were enriched in *Firmicutes* (10 OTUs overrepresented), particularly in *Streptococcus*, while they had a reduced richness in 10 OTUs from the most dominant phyla, *Bacteroidetes* (*Prevotella*), *Firmicutes* (*Veillonella*) and *Proteobacteria* (*Neisseria*) (Table 2, Supplementary Table S2). Additionally, the Aware-preserved samples clustered together more tightly along PC2 (Fig. 2), suggesting lower variability, and that relative differences between individuals in this group are obscured by this preservation technique.

Table 2. Bacterial taxa that changed in Aware preservation compared to freezing

Direction of change with Aware	Phylum	Order/family	Genus	No. of OTUs changed
Decrease	Proteobacteria	Campylobacterales		1
		Pasteurellaceae		3
		Neisseriaceae	Neisseria	2
		Burkholderiales		1
	Firmicutes	Veillonellaceae	Veillonella	3
		Streptococcaceae	Streptococcus	2
		Carnobacteriaceae	Granulicatella	1
		Fusobacteriaceae	Fusobacterium	1
	Bacteroidetes	Prevotellaceae	Prevotella	2
		Bacteroidales		2
	Actinobacteria	Actinomycetaceae	Actinomyces	1
Increase	Firmicutes	Streptococcaceae	Streptococcus	6
		Aerococcaceae	Abiotrophia	1
		Bacillales	Gemella	2
	Actinobacteria	Micrococcaceae	Rothia	1

Fig. 2. Principal components analysis of bacterial communities from oral samples from six Guahibo Amerindians, preserved either in Aware Messenger solution (red) or in liquid nitrogen (blue). Beta diversity was analysed using the UniFrac metric, in which distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from the 16S rRNA gene sequences from all communities being compared (Lozupone & Knight, 2005; Lozupone *et al.*, 2007).

DISCUSSION

This was a preliminary study conducted on only a few subjects, and at a sequencing depth sufficient to determine the general structure of the bacterial community at the supra-species level and inter-individual variability. The dominant phyla found in the oral mucosa of Amerindians were similar to those reported in buccal samples of non-Amerindians: *Firmicutes*, *Proteobacteria*, *Actinobacteria* (Huyghe *et al.*, 2008; Lazarevic *et al.*, 2009; Nasidze *et al.*, 2009a, b; Zaura *et al.*, 2009). With similar methodology to ours, in studies of non-Amerindians other authors found

variable numbers of bacterial phyla in saliva (Table 3). Across-studies comparisons have to be made with caution, due to differences in sequencing efforts, methodologies and oral sites studied (Engelbrektson *et al.*, 2010). When comparing bacteria from the oral mucosa in Amerindians with previous reports on non-Amerindians, we found similar numbers of families (30 in Amerindians and 33 in non-Amerindians), but with higher richness of genera in non-Amerindians (177) than in Amerindians (51) (Supplementary Table S3). Amerindians shared only 62 % of the families and 23 % of the genera with non-Amerindians (Fig. 3).

The Amerindians carried marker bacteria that were not reported in previous studies (Supplementary Table S3), such as soil bacteria Gp1 (*Acidobacteriaceae*) (Ausec *et al.*, 2009; Castro *et al.*, 2010), and the xylanolytic *Xylanibacter* (*Prevotellaceae*) (Ueki *et al.*, 2006), as well as *Phocoenobacter* (*Pasteurellaceae*), first described in a porpoise (Foster *et al.*, 2000), in addition to unidentified members of the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*.

Several methods, such as cooling, freezing, fixing, drying, freeze-drying and enzyme inhibitors, have been used to preserve samples (Brambilla *et al.*, 1992). Freezing is the most widely used sample preservation method for molecular studies, but it can complicate the logistics of sampling in remote settings. Sample preservation devices have been developed to replace freezing, including FTA paper (Whatman), RNeasy Protect Saliva Mini kit (Qiagen) and Aware Messenger solution. None of these devices had to our knowledge been evaluated in terms of preservation of sample DNA, and this study demonstrates that at least the Aware Messenger is not a good method for preserving nucleic acids if the goal is to perform a community-level analysis with stored DNA. However, Aware Messenger apparently selected for some OTUs that were

Table 3. Recent studies of human oral bacteria using culture-independent methods

Study	Subjects/lifestyle/sample	No. of subjects	OTU level	No. of bacterial phyla	Method
Paster <i>et al.</i> (2001)	Caucasian/urban/subgingival plaque	5 healthy, 26 diseased	415 species	9	PCR/cloning
Nasidze <i>et al.</i> (2009a)	Caucasian/urban/saliva	120 healthy	101 genera	13	
Bik <i>et al.</i> (2010)	6 Caucasian, 1 Afro-American, 2 Chinese, 1 Indian/urban/oral tissues and gingiva	10 healthy	247 species	9	
Huyghe <i>et al.</i> (2008)	Caucasian/urban/gingival fluid	2 healthy	9 genera	16	Microarray
Lazarevic <i>et al.</i> (2009)	Caucasian/urban/saliva and oropharyngeal	3 healthy	135 genera (V5)	12	Illumina
Keijser <i>et al.</i> (2008)	Caucasian/urban/saliva	71 healthy	3621 species in 31 genera (V6)	22	454 pyrosequencing
Nasidze <i>et al.</i> (2009b)	Caucasian/urban/saliva	12 healthy	79 genera (V1, V2)	12	
Zaura <i>et al.</i> (2009)	Caucasian/urban/dental surfaces, cheek, hard palate, tongue and saliva	3 healthy	78 genera (V5, V6)	8	
This study	Amerindian/rural/oral mucosa	6 healthy	693 species in 51 genera (V2)	10

Fig. 3. Venn diagram of the numbers of shared and exclusive oral bacterial families (a) and genera (b) in Amerindians (green circles) and non-Amerindians (violet circles).

undetected in the frozen swabs, such as *Lactobacillales*, *Lactobacillus*, *Dialister* and *Ruminococcaceae* (*Firmicutes*), *Actinomycetales* and *Bifidobacteriaceae* (*Acidobacteria*), although these may have been present in the Aware solution before adding the sample. However, if present in the Aware solution, we would expect the same species to have appeared in all samples preserved in Aware, which was not the case (Supplementary Table S2). In addition to DNA preservation, the Aware solution might have altered the growth capabilities of bacteria, enriching species that were able to survive or grow better. Aware Messenger solution is thus not a suitable alternative to freezing, since it affects the composition of the original sample relative to freezing.

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