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ORIGINAL ARTICLE

Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis

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AIM: Compare the microbial profiles on the tongue dorsum in patients with halitosis and control subjects in a UK population using culture-independent techniques.
MATERIALS AND METHODS: Halitosis patients were screened according to our recently developed recruitment protocol. Scrapings from the tongue dorsum were obtained for 12 control subjects and 20 halitosis patients. Bacteria were identified by PCR amplification, cloning and sequencing of 16S rRNA genes.
RESULTS: The predominant species found in the control samples were *Lysobacter*-type species, *Streptococcus salivarius*, *Veillonella dispar*, unidentified oral bacterium, *Actinomyces odontolyticus*, *Atopobium parvulum* and *Veillonella atypica*. In the halitosis samples, *Lysobacter*-type species, *S. salivarius*, *Prevotella melaninogenica*, unidentified oral bacterium, *Prevotella veroralis* and *Prevotella pallens* were the most commonly found species. For the control samples, 13–16 (4.7–5.8%) of 276 clones represented uncultured species, whereas in the halitosis samples, this proportion increased to 6.5–9.6% (36–53 of 553 clones). In the control samples, 22 (8.0%) of 276 clones represented potentially novel phylotypes, and in the halitosis samples, this figure was 39 (7.1%) of 553 clones.
CONCLUSIONS: The microflora associated with the tongue dorsum is complex in both the control and halitosis groups, but several key species predominate in both groups.
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Keywords: halitosis; bacteria; PCR; 16S rRNA; cloning; sequencing

Introduction

Halitosis, or oral malodour, is a non-life threatening but distressing condition that is relatively common in the adult population (Scully *et al*, 1994). However, the condition can have serious consequences socially and psychologically (Oho *et al*, 2001). Halitosis is primarily due to metabolic products generated by bacteria in the oral cavity, but may also arise as a result of systemic diseases (Greenman, 1999). The principal malodorous products of bacterial metabolism in the oral cavity are volatile sulphur compounds (VSCs), which account for approximately 90% of oral malodour (Tonzetich, 1971). The major VSCs found in oral malodour are hydrogen sulphide and methyl mercaptan, which are produced by bacterial metabolism of the sulphur-containing amino acids cysteine and methionine respectively (Persson *et al*, 1990). In some individuals, halitosis may be persistent and does not necessarily result from poor oral hygiene, and many halitosis sufferers are periodontally healthy (Bosy *et al*, 1994).

Studies on the tongue biofilm have been relatively few in number, compared with the significant number of investigations of dental plaque and the microflora associated with periodontal disease and dental caries. The tongue is known to harbour a very diverse flora at high cell density and is recognized as the major site of malodour generation in the oral cavity (De Boever and Loesche, 1995; Hartley *et al*, 1996). The organisms with the greatest malodour forming potential are Gram-negative anaerobes such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (previously known as *Bacteroides forsythus*), and *Fusobacterium nucleatum* (McNamara *et al*, 1972; De Boever and Loesche, 1995; Quirynen *et al*, 1999). Diamines, such as cadaverine, and volatile organic acids may also contribute to oral malodour to a lesser extent (Kostelc *et al*, 1980; Goldberg *et al*, 1994). Studies on human volunteers have demonstrated a correlation between the level of oral malodour and numbers of Gram-negative anaerobes and sulphide-producing organisms on the

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tongue surface (Greenman, 1999). Compounds such as chlorhexidine gluconate, metronidazole and zinc have been shown to be effective at reducing oral malodour, presumably by decreasing the bacterial load associated with the tongue (De Boever and Loesche, 1995; Waler, 1997; Hartley *et al*, 1999).

Much of the previous research into the causes of halitosis has concentrated on the characterization of the tongue microflora using conventional microbiological culture methods. However, it has been estimated that approximately 50% of the oral microflora is uncultivable (Socransky *et al*, 1963). It can be hypothesized that uncultivable or perhaps even novel species contribute to the development of halitosis. A recent study used culture-independent methods to more fully characterize the microflora associated with the tongue biofilm of a small number of North American subjects with and without halitosis (Kazor *et al*, 2003). The purpose of our study was to characterize the tongue microflora in a much larger cohort of subjects from the UK (12 control and 20 halitosis) using culture-independent methods.

Materials and methods

Study subjects

Patients complaining of halitosis were recruited to the study principally by means of a newspaper advertisement. A rigorous screening protocol recently described by our group (Donaldson *et al*, 2007) was used to identify subjects suffering from halitosis which was not related to chronic gingivitis, chronic periodontitis or pathology of the oral mucosa. This screening protocol applied the following exclusion criteria to enrolled subjects: poor oral hygiene; generalized chronic gingivitis or periodontitis; pathology of the oral mucous membranes or attached gingivae; respiratory tract diseases; diabetes mellitus, kidney, liver or stomach disorders; HIV/AIDS, Sjögren's syndrome; antibiotic therapy in the preceding 4 weeks; prescribed medication that can cause xerostomia; edentulousness; smoking. For 48 h prior to assessment subjects were asked to avoid eating foods containing garlic, onions and strong spices, and to refrain from consuming alcohol and using mouthwashes. On the morning of assessment, they were asked to refrain from drinking coffee, using mint-containing products and wearing heavily scented products. Subjects were asked to have a light breakfast no less than 2 h before the assessment and to brush their teeth with water in order to remove plaque deposits and food debris.

Halitosis was confirmed by means of organoleptic assessment (Yaegaki and Coil, 2000) and VSC levels measured using a Halimeter (Interscan, Chatsworth, CA, USA). A four-point organoleptic scale (0, no malodour; 1, slight but non-objectionable odour; 2, objectionable odour; 3, very strong odour) was used (Schmidt *et al*, 1978). VSC levels of at least 200 ppb or an organoleptic score of 2 or 3 was indicative of halitosis, whereas VSC levels <200 ppb or an organoleptic score of 0 or 1 indicated the absence of halitosis. Twenty subjects with halitosis and 12 subjects without halitosis were recruited to the study. The clinical parameters for these two groups are shown in Table 1.

Table 1 Organoleptic and Halimeter scores for control subjects and halitosis patients

Sample no.	*Organoleptic score*	*Halimeter score*
Control subjects ($n = 12$)		
C1	1	146
C2	1	96
C3	1	111
C4	1	109
C5	1	156
C6	1	173
C7	1	115
C8	1	196
C9	1	106
C10	1	123
C11	1	130
C12	1	155
Halitosis patients ($n = 20$)		
H1	2	130
H2	3	936
H3	2	285
H4	2	173
H5	2	187
H6	2	261
H7	2	314
H8	2	496
H9	2	294
H10	2	414
H11	2	179
H12	2	327
H13	2	335
H14	2	155
H15	2	207
H16	2	318
H17	2	238
H18	2	368
H19	2	193
H20	2	174

Sample collection

A sample was collected from the dorsum of the tongue anterior to the circumvallate papillae by vigorous brushing using a sterile, tapered wire brush (Medical Wire and Equipment Ltd, Corsham, UK) and placed into 200 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Sample processing

Samples were mixed for 30 s and a crude bacterial DNA extract was produced from each sample by adding 3 μl of achromopeptidase (20 U μl^{-1} in 10 mM Tris–HCl, 1 mM EDTA, pH 7.0) to 100 μl of sample. Samples were incubated at 56°C for 30 min, boiled for 5 min and stored at −70°C until required.

PCR amplification of bacterial 16S rRNA genes

Universal primers were used to amplify bacterial 16S rRNA genes. The primer sequences were 5′-AGA GTT TGA TCM TGG CTC AG-3′ (27f; *Escherichia coli* nt 8–27) (Lane, 1991) and 5′-GGG CGG WGT GTA CAA GGC-3′ (1387r; *E. coli* nt 1387–1404 (Marchesi *et al*,

1998); where M = C + A and W = A + T, and give an expected amplification product of approximately 1400 bp. Primers were synthesized commercially (MWG Biotech, Milton Keynes, UK). All PCR reactions were carried out in a total volume of 50 μl, comprising 5 μl of crude bacterial DNA extract and 45 μl of reaction mixture containing 1x PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 1.0 U *Taq* DNA polymerase (Promega Corporation, Southampton, UK), 0.2 mM dNTPs (GE Healthcare, Little Chalfont, UK), and each primer at a concentration of 0.2 μM. The PCR cycling conditions comprised an initial denaturation step at 94°C for 5 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min and extension at 72°C for 1 min, and finally an extension step at 72°C for 10 min.

PCR quality control
When carrying out PCR, stringent procedures were employed to prevent contamination, as previously described (Riggio *et al*, 2000). Negative and positive controls were included with each batch of samples being analyzed. The positive control comprised a standard PCR reaction mixture containing 10 ng of *E. coli* genomic DNA instead of sample, whereas the negative control contained sterile water instead of sample. One negative control was used for every three samples analyzed. Ten microlitres of each PCR product was electrophoresed on a 2% agarose gel, and amplified DNA detected by staining with ethidium bromide (0.5 μg ml^{-1}) and visualization under ultraviolet light.

Cloning of 16S rRNA PCR products
PCR products were cloned into pCR2.1-TOPO cloning vector using the TOPO TA Cloning Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene inserts
Following cloning of the 16S rRNA gene product amplified by PCR for each sample, 50 clones from each generated library were randomly selected. The 16S rRNA gene insert from each clone was amplified by PCR using the primer pair 5′-GCT ATT ACG CCA GCT GGC GAA AGG GGG ATG TG-3′ (M13FAP) and 5′-CCC CAG GCT TTA CAC TTT ATG CTT CCG GCA CG-3′ (M13RAP). The M13FAP binding site is located 52 bp downstream of the M13 Forward primer-binding site, and the M13RAP binding is located 39 bp upstream of the M13 Reverse primer-binding site, in the pCR2.1-TOPO vector.

Restriction enzyme analysis
Selected clones from the libraries were subjected to restriction enzyme analysis with *Rsa*I and *Mnl*I. Approximately 0.5 μg of each PCR product was separately digested in a total volume of 15 μl with 2.0 U *Rsa*I (Promega) and 2.0 U *Mnl*I (Helena Biosciences, Sunderland, UK) at 37°C for 3 h. Restriction fragments were visualized by agarose gel electrophoresis as described above. For each library, clones were initially sorted into distinct groups on the basis of restriction profiles obtained with *Rsa*I. Further discrimination was obtained by digestion of clones with *Mnl*I, a restriction enzyme that is highly efficient at generating unique bacterial 16S rRNA fingerprints, which resulted in the identification of additional distinct restriction fragment length polymorphism (RFLP) groups.

DNA sequencing
The 16S rRNA gene of a single, representative clone from each group identified by restriction enzyme analysis was sequenced. Sequencing reactions were performed with the Fermentas Life Sciences CycleReader™ Auto DNA Sequencing Kit (Helena Biosciences) and IRD800-labelled 357f sequencing primer (Lane, 1991) on a Primus96 DNA thermal cycler (MWG Biotech) using the following cycling parameters: (i) initial denaturation at 95°C for 30 s; (ii) 10 s at 95°C, 30 s at 57°C and 30 s at 70°C, for 20 cycles and (iii) 10 s at 95°C and 30 s at 70°C for 15 cycles. Six microlitres of formamide loading dye was added to each reaction mixture following thermal cycling. 1.5 μl of each denatured sequencing reaction mixture was run on a LI-COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech) according to the manufacturer's instructions.

Sequence analysis
Sequence data were compiled using LI-COR Base ImagIR 4.0 software, converted to FASTA format and analysed for chimeric forms using the Chimera-CHECK 2.7 programme from the Ribosomal Database Project II (Maidak *et al*, 2000). Sequences were then compared with 16S rRNA gene sequences from public sequence databases Genbank and EMBL using the advanced gapped BLAST program, version 2.1. The program was subsequently run through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Clone sequences demonstrating at least 98% identity with a public database sequence were considered to be of the same species as the highest score matching sequence. Sequences with $<98\%$ identity with public database sequences were tentatively classified as potentially novel phylotypes.

Results

The organoleptic scores and Halimeter readings obtained for each of the subjects analyzed in the study are shown in Table 1. Fifty clones were randomly selected from each clone library (12 control and 20 halitosis subjects) and subjected to restriction enzyme analysis. Since many RFLP groups contained multiple clones with the same restriction profiles, in order to avoid sequencing redundancy a single representative clone from each group was sequenced. In order for a clone sequence to be deemed satisfactory for analysis, at least 500 bp of sequence had to be available for BLAST analysis with no evidence of chimeric sequences. Sequence data were obtained for a total of 276 clones

Table 2 Bacterial species identified by 16S rRNA gene sequencing (with sequence identities of at least 98%) on the tongue dorsum of 12 control subjects

Bacterial species	*No. of clones [% of total] n = 276*	*No. of samples in which present*
Abiotrophia para-adiacens	2 [0.7]	2
Actinomyces genomosp.	1 (1) [0.4–0.7]	1–2
Actinomyces graevenitzii	**1 [0.35]**	**1**
Actinomyces lingnae	6 (1) [2.2–2.5]	6
Actinomyces meyeri	2 [0.7]	2
Actinomyces odontolyticus	8 (1) [2.9–3.3]	5–6
Actinomyces sp. oral clone	**3 (1) [1.1–1.4]**	2–3
Actinomyces (undifferentiated)[a]	3 [1.1]	3
Aeromonas hydrophila	**1** [0.4]	**1**
Atopobium parvulum	9 [3.3]	4
Capnocytophaga granulosa	1 [0.4]	1
Capnocytophaga sp. oral clone	3 [1.1]	3
Eubacterium sp. oral clone	1 [0.4]	1
Fusobacterium nucleatum subsp. *polymorphum*	1 [0.4]	1
Fusobacterium periodonticum	**3 [1.1]**	**2**
Haemophilus influenzae	**1 [0.4]**	**1**
Haemophilus parainfluenzae	7 (2) [2.5–3.3]	3
Haemophilus paraphrophilus	**1 (2) [0.4–1.1]**	**1–2**
Lachnospiraceae oral clone[b]	**1 [0.4]**	**1**
Lysobacter-type sp.[c]	32 (1) [11.6–12.0]	9–10
Megasphaera micronuciformis	1 [0.4]	1
Micrococcus mucilaginosus	1 [0.4]	1
Oribaculum catoniae	**1 [0.4]**	**1**
Porphyromonas sp. oral clone	7 [2.5]	4
Prevotella melaninogenica	5 [1.8]	4
Prevotella oulora	2 [0.7]	1
Prevotella salivae	5 [1.8]	4
Prevotella sp. oral clone	8 [2.9]	4
Prevotella veroralis	2 [0.7]	2
Prevotellaceae bacterium[b]	2 [0.7]	7
Rothia sp.	**1 [0.4]**	**1**
Selenomonas flueggei-like sp.	**1 [0.4]**	**1**
Streptococcus genomosp.	**2 (1) [0.7–1.1]**	**2–3**
Streptococcus infantis	2 (1) [0.7–1.1]	1–2
Streptococcus mitis	4 [1.4]	3
Streptococcus oralis	2 [0.7]	2
Streptococcus parasanguis	4 (2) [1.4–2.2]	3
Streptococcus pneumoniae	2 [0.7]	2
Streptococcus salivarius	16 [5.8]	5–7
Streptococcus sanguinis	2 (2) [0.7–1.4]	2
Streptococcus sp. oral clone/strain	6 (3) [2.2–3.3]	3–4
Streptococcus (undifferentiated)[a]	11 [4.0]	6
Terrahaemophilus aromaticivorans	7 [2.5]	3
TM7 phylum sp. oral clone	2 [0.7]	2
Treponema sp.	**1 [0.4]**	**1**
Uncultured bacterium	1 [0.4]	1
Uncultured firmicute	**1 [0.4]**	**1**
Uncultured human oral bacterium	1 (1) [0.4–0.7]	1–2
Uncultured *Stenotrophomonas* sp.	**1 [0.4]**	**1**
Uncultured *Veillonella* sp. clone	7 (2) [2.5–3.3]	4
Unidentified oral bacterium	11 (1) [4.0–4.3]	6
Veillonella atypica	8 (3) [2.9–4.0]	6–7
Veillonella caviae	3 (3) [1.1–2.2]	2–4
Veillonella dispar	14 (1) [5.1–5.4]	5
Veillonella parvula	3 [1.1]	2
Veillonella sp. oral clone	(1) [0.0–0.4]	0–1
Veillonella (undifferentiated)[a]	3 [1.1]	3
Unknown (undifferentiated)[d]	2 [0.7]	2

() BLAST results indicate identity as being either of two possible species, usually of the same genus.
Bold type indicates species unique to the normal samples.
Where clones are identified as being one of two possible species, a frequency range is given.
[a]Three or more possible species (same genera).
[b]Family.
[c]Includes *Lysobacter* sp., *Lysobacter enzymogenes*, iron-oxidising lithotroph ES-1 and *Xanthomonas* sp.
[d]Three or more possible species (at least two different genera).

for the normal group and 553 clones for the halitosis group. Generally, 500–800 bp of DNA sequence were obtained for each clone. The bacterial species identified, and the frequency of occurrence of clones representing each species, is shown in Tables 2 and 3 for the control and halitosis samples respectively. Where BLAST results indicated identification of a clone as being one of two possible species, a frequency range is given.

Species distribution

Based upon the 276 clones from 12 samples, the most prevalent cultivable species identified in the control samples were: *Lysobacter*-type species (11.6–12.0% of clones, found in nine to 10 samples); *Streptococcus salivarius* (5.8% in seven samples); *Veillonella dispar* (5.1–5.4% in five samples); unidentified oral bacterium (4.0–4.3% in six samples); *Actinomyces odontolyticus* (2.9–3.3% in five to six samples); *Atopobium parvulum* (3.3% in four samples); *Veillonella atypica* (2.9–4.0% in six to seven samples); *Streptococcus* sp. oral clone/strain (2.2–3.3% in three to four samples); *Prevotella* sp. oral clone (2.9% in four samples); *Porphyromonas* sp. oral clone (2.5% in four samples).

Based upon sequence data obtained for 553 clones from 20 halitosis samples, the most prevalent cultivable species were: *Lysobacter*-type species (7.2% in 13 samples); *S. salivarius* (6.1–6.7% in 12 samples); *Prevotella melaninogenica* (5.1% in 11 samples); unidentified oral bacterium (4.3–5.8% in 13 samples); *Prevotella veroralis* (2.9% in nine samples); *Prevotella pallens* (2.7% in nine samples); *A. parvulum* (2.7% in nine samples); *Streptococcus mitis* (2.5–3.3% in seven to eight samples); *A. odontolyticus* (2.5% in 10 samples); *Veillonella parvula* (1.8–2.0% in four to five samples); *Porphyromonas* sp. oral clone (1.8% in six samples); *Streptococcus oralis* (1.8% in six samples).

Uncultured species

For the control samples, 13–16 (4.7–5.8%) of 276 clones, present in six samples, represented uncultured species (including the TM7 phylum). In the halitosis samples, this proportion increased to 6.5–9.6% (36–53 of 553 clones), being present in 16 samples. Uncultured *Veillonella* sp. were the most frequently found uncultured species in both the control samples (2.5–3.3% in four samples) and the halitosis samples (2.5–3.4% in nine to 10 samples).

Table 3 Bacterial species identified by 16S rRNA gene sequencing (with sequence identities of at least 98%) on the tongue dorsum of 20 subjects with halitosis

Bacterial species	*No. of clones [% of total] n = 553*	*No. of samples in which present*
Abiotrophia elegans	**2 [0.4]**	**1**
Abiotrophia para-adiacens	3 (2) [0.5–0.9]	3–4
Actinomyces genomo sp.	2 [0.4]	1
Actinomyces lingnae	3 [0.5]	3
Actinomyces meyeri	3 [0.5]	3
Actinomyces odontolyticus	14 [2.5]	10
Actinomyces sp.	3 [0.5]	2
Actinomyces (undifferentiated)[a]	7 [1.3]	5
Atopobium parvulum	15 [2.7]	9
Bacteroidales strain	**1 [0.2]**	**1**
Bacteroides forsythus (*Tannerella forsythensis*) oral clone	**2 [0.4]**	**1**
Bulleidia (*Solobacterium*) *moorei*	**6 [1.1]**	**5**
Capnocytophaga gingivalis	**2 [0.4]**	**2**
Capnocytophaga granulosa	2 [0.4]	1
Capnocytophaga sp. oral strain	1 [0.2]	1
Capnocytophaga sputigena	**4 [0.7]**	**1**
Escherichia coli	**1 [0.2]**	**1**
Eubacterium sp.	**1 [0.2]**	**1**
Eubacterium sp. oral clone	2 [0.4]	2
Firmicutes[b] sp. oral strain/clone	3 [0.5]	3
Fusobacterium nucleatum subsp. *polymorphum*	3 [0.5]	3
Fusobacterium sulci	**1 [0.2]**	**1**
Gemella haemolysans	**1 [0.2]**	**1**
Gemella sanguinis	**1 [0.2]**	**1**
Granulicatella adiacens	**(1) [0.0–0.2]**	**0–1**
Haemophilus parainfluenzae	4 [0.7]	2
Human intestinal firmicute	**1 [0.2]**	**1**
Human oral bacterium	**1 (1) [0.2–0.4]**	**1–2**
Lachnospiraceae bacterium[c]	**1 [0.2]**	**1**
Lysobacter-type sp.[d]	40 [7.2]	13
Megasphaera micronuciformis	7 [1.3]	4
Megasphaera sp. oral clone	**2 [0.4]**	**1**
Micrococcus mucilaginosus	1 [0.2]	1
Mogibacterium neglectum	**1 [0.2]**	**1**
Neisseria perflava	**4 [0.7]**	**4**
Neisseria subflava	**5 [0.9]**	**5**
Porphyromonas sp. oral clone	10 [1.8]	6
Prevotella melaninogenica	28 [5.1]	11
Prevotella oulora	1 [0.2]	1
Prevotella pallens	**15 [2.7]**	**9**
Prevotella salivae	8 [1.4]	7
Prevotella shahii	**2 [0.4]**	**1**
Prevotella sp. oral clone	9 [1.6]	4
Prevotella tannerae	**3 [0.5]**	**3**
Prevotella veroralis	16 [2.9]	9
Prevotellaceae bacterium	4 [0.7]	2
Rothia dentocariosa	**1 [0.2]**	**1**
Streptococcus australis	**2 [0.4]**	**2**
Streptococcus cristatus	**1 [0.2]**	**1**
Streptococcus infantis	5 (1) [0.9–1.1]	4
Streptococcus mitis	14 (4) [2.5–3.3]	7–8
Streptococcus oralis	10 [1.8]	6
Streptococcus parasanguis	8 (5) [1.4–2.4]	5–7
Streptococcus pneumoniae	1 (3) [0.2–0.7]	1–3
Streptococcus salivarius	34 (3) [6.1–6.7]	12
Streptococcus sanguinis	3 (2) [0.5–0.9]	3–4
Streptococcus sp. oral clone/strain	9 (1) [1.6–1.8]	4–5
Streptococcus (undifferentiated)[a]	7 [1.3]	5
Terrahaemophilus aromaticivorans	3 [0.5]	2
TM7 phylum oral clone	1 [0.2]	1
Uncultured bacterium clone	0 (2) [0.0–0.4]	0–2
Uncultured *Eubacterium* sp.	**1 (1) [0.2–0.4]**	**1**
Uncultured human oral bacterium	10 (5) [1.8–2.7]	4–8
Uncultured *Megasphaera* sp.	**2 [0.4]**	**2**
Uncultured *Prevotella* sp.	**7 [1.3]**	**3**
Uncultured rumen bacterium	**1 [0.2]**	**1**
Uncultured *Streptococcus* sp.	**(4) [0.0–0.7]**	**0–2**
Uncultured *Veillonella* sp.	14 (5) [2.5–3.4]	9–10
Unidentified oral bacterium	24 (8) [4.3–5.8]	13
Veillonella atypica	23 (10) [4.2–6.0]	11
Veillonella caviae	1 (7) [0.2–1.4]	1–5
Veillonella dispar	35 (2) [6.3–6.7]	13–14
Veillonella parvula	10 (1) [1.8–2.0]	4–5
Veillonella sp. oral clone	0 (7) [0.0–1.3]	0–6
Veillonella (undifferentiated)[a]	1 [0.2]	1
Unknown (undifferentiated)[e]	2 [0.4]	2

() BLAST results indicate identity as being either of two possible species, usually of the same genus.
Bold type indicates species unique to the halitosis samples.
Where clones are identified as being one of two possible species, a frequency range is given.
[a]Three or more possible species (same genera).
[b]Division.
[c]Family.
[d]Includes *Lysobacter* sp., *Lysobacter enzymogenes*, iron-oxidising lithotroph ES-1 and *Xanthomonas* sp.
[e]Three or more possible species (at least two different genera).

Potentially novel phylotypes

Only sequence alignments for which the sequence identity was <98% and where at least 500 bases were available for alignment were included in this analysis. In the control samples, 22 (8.0%) of 276 clones represented potentially novel phylotypes, being present in nine samples; in the halitosis samples, 39 (7.1%) of 553 clones represented potentially novel phylotypes, being present in 15 samples. Details of the 20 clones representing phylotypes with the lowest percentage identities to known sequences in the public access databases are shown in Table 4.

Species unique to the control and halitosis samples

Species that were found only in the control or halitosis samples are highlighted in Tables 2 and 3 respectively. Thirty species were unique to the halitosis samples, whereas only 14 species were unique to the control samples. Of particular note is the finding that *P. pallens* is unique to the halitosis samples, being found in nine halitosis samples and representing 2.7% of the total clones analyzed. *Bulleidia* (*Solobacterium*) *moorei* was the next most prevalent species only found in the halitosis samples, being found in five samples and representing 1.1% of the clones analyzed.

Discussion

In this study, molecular cloning and sequence analysis of bacterial 16S rRNA gene sequences was used to identify bacteria present on the tongue dorsum of 20 subjects with halitosis and 12 controls. This study represents the

Table 4 Details of a selection of sequenced clones representing potentially novel species

Sample no. (clone no.)	*Sequenced bases available for BLAST*	*Matching bases*	*Sequence identity (%)*	*Accession no.*	*Most closely matched bacterial species*
H1 (08)	666	535/573	93.4	AF439641	*Veillonella atypica*
H1 (15)	690	602/641	93.9	AF201991	Uncultured human oral bacterium A43
H1 (29)	593	537/571	94.0	AF439641	*V. atypica*
H3 (02)	669	574/607	94.6	X84007	*V. atypica*
H3 (08)	588	514/551	93.3	AJ320168	Uncultured *Veillonella* sp.
H5 (04)	715	614/654	93.9	L06168	*Neisseria flavescens*
H5 (13)	795	622/665	93.5	AF479578	*Neisseria subflava*
H11 (08)	697	593/629	94.2	AJ551156	*Pseudomonas* sp. An18
H12 (01)	861	719/754	95.4	X82823	*Oribaculum catoniae*
H12 (04)	773	667/716	93.2	AB108825	*Prevotella shahii*
H12 (20)	770	567/606	91.3	AB034127	Uncultured rumen bacterium 4C28d-18
H13 (07)	647	550/589	93.4	AB108825	*P. shahii*
H13 (33)	686	534/601	88.9	AB034021	Uncultured rumen bacterium 4C0d-8
H16 (03)	765	637/670	95.1	AF439641	*V. atypica*
H16 (39)	781	671/707	94.9	AB108826	*Prevotella salivae*
H17 (02)	746	662/695	95.3	L16470	*Prevotella melaninogenica* ATCC 43982
H18 (11)	716	622/669	93.0	AY323525	*P. melaninogenica*
C5 (18)	669	507/550	92.2	AF385510	*Eubacterium* sp. oral clone DO016
C6 (13)	676	555/582	95.3	L16473	*Prevotella veroralis* ATCC 33779
C10 (39)	713	598/632	94.6	AF003928	*Streptococcus sanguinis*

The 20 clones with the lowest percentage identities to known sequences in public access databases are shown.

most extensive microbial analysis performed to date on the tongue dorsum in subjects with halitosis. Since a strict screening protocol was employed to eliminate other factors that may be causing oral malodour (Donaldson *et al*, 2006), the data presented represent an insight into the bacteria on the dorsum of the tongue that may be involved in genuine physiological halitosis.

Some studies which have used 16S rRNA gene sequencing to identify bacteria in a relatively small number of clinical specimens have adopted the approach of sequencing approximately 50 clones from each library generated per sample (Paster *et al*, 2002; Kazor *et al*, 2003). Due to the relatively large number of samples analyzed in our study, we sought to minimize sequencing of identical clones by screening using restriction RFLP analysis, and sequencing a single representative clone from each RFLP group. This approach has been successfully used in many studies to avoid sequencing redundancy and to estimate bacterial diversity within clinical specimens (Rossetti *et al*, 2003; Verhelst *et al*, 2004; Shinzato *et al*, 2005).

A key finding of our study was the greater microbial diversity on the tongue dorsum of subjects with halitosis compared with controls. Uncultured species increased from between 4.7 and 5.8% of the total clones analysed in the control samples (in six of 12 samples) to between 6.5 and 9.6% in the halitosis samples (in 16 of 20 samples). Potentially novel phylotypes were present at similar levels in both sample groups, representing 8.0% of clones (in nine of 12 samples) and 7.1% of clones (in 15 of 20 samples) in the control and halitosis samples, respectively, although sequencing of the entire 16S rRNA gene would be required for full confirmation. Excluding *Lysobacter*-type species, the most prevalent genera in both sample types were *Streptococcus*, *Veillonella* and *Prevotella* species. However, while the proportion of *Streptococcus* and *Veillonella* species was similar in both groups, the level of *Prevotella* species was much higher in the halitosis samples [89 (16.1%) of 553 clones; in 17 of 20 samples] than in the control samples [22 (8.0%) of 276 clones; in nine of 12 samples]. This twofold increase in the level of *Prevotella* species included an increase in *P. melaninogenica* from 5 (1.8%) of 276 clones (in four of 12 control samples) to 28 (5.1%) of 553 clones (in 11 of 20 halitosis samples) and, significantly, by the appearance of *P. pallens* in the halitosis samples [15 (2.7%) of 553 clones; in nine of 20 samples], a species which was absent in the control samples. Interestingly, Kazor *et al* (2003) identified this species in only one of six halitosis samples and none of five normal samples analyzed.

Overall, the types of bacteria found between the study of Kazor *et al* (2003) and our present study were very similar, but there were differences in the predominant species found in the halitosis and control groups. The predominant species in the control group in that study were *S. salivarius*, *Rothia mucilaginosa* (*Stomatococcus mucilaginosus*) and an uncharacterized cultivable species of *Eubacterium* (strain FTB41). In the present study, the predominant species in the control group were *Lysobacter*-type species (11.6–12.0% of clones; in nine to 10 of 12 samples), *S. salivarius* (5.8% of clones; in five to seven of 12 samples) and *V. dispar* (5.1–5.4% of clones; in five of 12 samples). In the study of Kazor *et al* (2003), *S. salivarius* comprised 12 to 41% of the clones analysed in each control sample, compared with the much lower range of 3.1–14.3% in the current study. *Lysobacter*-type species formed the most prevalent group in both the halitosis and control samples in our study, including *Lysobacter* sp. (principally strain C3), *Lysobacter enzymogenes*, iron-oxidising lithotroph ES-1 and *Xanthomonas* sp. Sullivan *et al* (2003) described the evolutionary relationship between members of the *Lysobacter* clade.

Lysobacter sp. strain C3 was initially identified as *Stenotrophomonas maltophilia* (Giesler and Luen, 1998). This organism was first reported as an environmental species but is now known to be an emerging nosocomial pathogen, implicated in a range of human infections, including septic arthritis in an AIDS patient (Belzunegui *et al*, 2000). It can adhere avidly to medical implants and catheters, forming a biofilm (De Oliveira-Garcia *et al*, 2003). *S. maltophilia* has been identified by 16S rRNA gene sequencing as the predominant species in advanced noma lesions (Paster *et al*, 2002) and has been isolated from a case of acute necrotizing gingivitis in an immunocompromised individual (Miyairi *et al*, 2005). Whether *Stenotrophomonas*/*Lysobacter* species are natural members of the oral flora or are merely transient would require further study.

The molecular results obtained correlated with the microbiological culture data on the same samples to a limited extent (Donaldson *et al*, 2005). The most frequently found genera in both groups using microbiological culture were *Veillonella*, *Prevotella* and *Fusobacterium*. Using molecular techniques, the most frequently found genera (excluding the *Lysobacter*-type species) were *Streptococcus*, *Veillonella*, *Prevotella* and *Actinomyces* in both test groups. Members of the *Streptococcus* genus were not identified by culture methods since the protocol used sought to identify only Gram-negative species. However, the predominance of *Veillonella* and *Prevotella* species in both test groups using both methods was demonstrated, although *Fusobacterium* species were rarely identified using molecular methods.

Whilst the results of the current study have provided additional information on the microflora of the dorsal surface of the tongue, it was not possible to examine the production of VSCs by the organisms identified. However, in a recent study Washio *et al* (2005) identified H_2S-producing bacteria in the tongue biofilm of five subjects with no/low oral malodour and five subjects with oral malodour. The numbers of total bacteria and H_2S-producing bacteria in the malodour group were significantly greater than those in the no/low odour group, suggesting a quantitative, rather than qualitative effect. The authors identified isolates obtained by microbiological culture through 16S rRNA gene sequencing, and reported that *Veillonella*, *Actinomyces* and *Prevotella* species were the most prevalent genera in both groups, a finding corroborated by our study. Further similarities between the results reported by Washio *et al* (2005) and our own study were that qualitatively the bacterial composition was similar between the halitosis and control groups, and that key periodontal pathogens such as *P. gingivalis* and *P. intermedia* were not detected.

In conclusion, our study has demonstrated that the microflora associated with the tongue dorsum is complex in both the control and halitosis groups. The molecular approach used in this study to identify bacteria has shown that several fastidious, uncultivable and potentially novel species are present on the tongue dorsum in both control subjects and in those with halitosis. However, further studies are required on the physiological mechanisms involved in production of VSCs by the organisms identified, before a full understanding of the increased production of oral malodour in subjects with halitosis will be reached.

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