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Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodour

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The aims of this study were to identify hydrogen sulfide (H_2S)-producing bacteria among tongue biofilm microflora and to investigate the relationship between bacterial flora and H_2S levels in mouth air. Oral malodour levels in 10 subjects (age 21–56 years) were assessed by gas chromatography, and Breathtron and organoleptic scores. Based on these assessments, subjects were divided into two groups: an odour group and a no/low odour group. Tongue coatings were sampled and spread onto Fastidious Anaerobe Agar plates containing 0·05 % cysteine, 0·12 % glutathione and 0·02 % lead acetate, and were then incubated anaerobically at 37 °C for 2 weeks. Bacteria forming black or grey colonies were selected as H_2S-producing phenotypes. The numbers of total bacteria ($P < 0·005$) and H_2S-producing bacteria ($P < 0·05$) in the odour group were significantly larger than those in the no/low odour group. Bacteria forming black or grey colonies (126 isolates from the odour group; 242 isolates from the no/low odour group) were subcultured, confirmed as producing H_2S and identified according to 16S rRNA gene sequencing. Species of *Veillonella* (38·1 % in odour group; 46·3 % in no/low odour group), *Actinomyces* (25·4 %; 17·7 %) and *Prevotella* (10·3 %; 7·8 %) were the predominant H_2S-producing bacteria in both the odour and no/low odour groups. These results suggest that an increase in the number of H_2S-producing bacteria in the tongue biofilm is responsible for oral malodour, although the bacterial composition of tongue biofilm was similar between the two groups.

INTRODUCTION

Oral malodour is foul-smelling breath exhaled from the oral cavity and is due to metabolic products of bacteria in the oral cavity but can also be caused by systemic diseases, such as gastrointestinal disorders, hepatic diseases and diabetes, ingestion of certain foods and smoking (Greenman, 1999). Approximately 90 % of oral malodour is believed to originate from foul-smelling gases, such as volatile sulfur compounds (VSCs), produced by oral bacteria in the oral cavity (Ayers *et al.*, 1998; Scully *et al.*, 1994). The major components of VSCs in oral malodour are hydrogen sulfide (H_2S), methyl mercaptan and dimethyl sulfide (Kleinberg & Westbay, 1990). These VSCs are produced through bacterial metabolism of sulfur amino acids such as cysteine and methionine (Persson *et al.*, 1990).

VSC-producing bacteria are present at various sites in the oral cavity, particularly on the dorsum of the tongue, where they have easy access to nutrients, such as saliva, desquamated epithelium and food debris (Roldan *et al.*, 2003). Therefore, the coating on the dorsum of the tongue is widely recognized as a major source of VSCs (De Boever & Loesche, 1995; Nakano *et al.*, 2002; Rosenberg, 1996; Yaegaki & Sunada, 1992a, b).

Most previous studies have focused on the relationship between oral malodour and salivary or dental plaque bacteria (Awano *et al.*, 2002; Paryavi-Gholami *et al.*, 1999; Persson *et al.*, 1990). Following work by Gordon *et al.* (1966), studies have been conducted to analyse bacteria in the tongue biofilm, but most have targeted a limited number of bacterial species (Frisken *et al.*, 1990; Miyake *et al.*, 1991; van Winkelhoff *et al.*, 1986). Comprehensive analyses of tongue biofilm microflora using culture methods or molecular biological methods have recently been reported (Hartley *et al.*, 1996, 1999; Kazor *et al.*, 2003; Milnes *et al.*, 1993). Due to its complexity, however, the characteristics of tongue biofilm microflora and its relationship with oral malodour remain unclear (Hartley *et al.*, 1996, 1999; Kazor *et al.*, 2003).

Paryavi-Gholami *et al.* (1999) reported the isolation and identification of H_2S-producing bacteria from the saliva of children, using agar plates including lead acetate, and discussed the relationship between salivary H_2S-producing bacteria and oral malodour. Applying their methods, the aims of this study were to isolate and identify H_2S-producing

Abbreviation: VSC, volatile sulfur compound.

bacteria from the tongue biofilm using molecular biological methods, such as PCR and DNA sequencing, and to determine any relationships between the number or type of H_2S-producing bacteria and oral malodour.

METHODS

Subjects. Ten subjects (five females and five males; age, mean ± SD, 36·3 ± 11·1 years; range, 21–56 years) were selected for this study. Informed consent was obtained from each subject. All subjects were patients who visited Tohoku University Dental Hospital complaining of halitosis. They had no systemic disease and received no antibiotic therapy for at least 3 months. On the first visit, an assessment of oral malodour and observable tongue coating, a clinical oral examination and sampling of tongue biofilm were performed as described below.

Oral malodour assessment. Level of oral malodour was assessed by gas chromatography (GC; Shimadzu GC-7A, Kyoto), and Breathtron (New Cosmos Electric) and organoleptic scoring. Breathtron is a portable monitor with a zinc-oxide thin film semiconductor sensor specific to VSCs (Shimura *et al.*, 1996). All subjects were asked not to brush, rinse or smoke immediately prior to the assessment, and not to eat and drink for at least 2 h before assessment. GC analysis was carried out in duplicate. After closing the lips for 1 min, 5 ml of mouth air was obtained with a gastight syringe and immediately injected into the GC equipment. Standard samples of H_2S and methyl mercaptan (Sumitomo Seika Chemicals) were used as controls. Breathtron analysis was also performed in duplicate. Organoleptic scores were assessed by three judges immediately after closing the lips for 30 s. Scores were given as follows: 0, no malodour; 1, slight malodour; 2, clearly noticeable malodour; 3, strong malodour; and 4, extremely strong malodour.

Clinical oral examination. All subjects were examined for dental caries, plaque accumulation by O'Leary plaque control record index (O'Leary *et al.*, 1972) and probing depth using a periodontal pocket probe. No subjects lacked numerous teeth, wore dentures or exhibited severe caries, severe gingivitis, periodontitis or any other oral disease associated with oral malodour.

Observable tongue coating assessment. Thickness and extent of tongue coating were estimated by the naked eye according to the method of Nara (1977). Both thickness and extent of tongue coating were scored as 0, 1, 2 or 3, and then the thickness score and the extent score were multiplied.

Sampling of tongue biofilm. In order to collect tongue biofilm, an area of 1 cm^2, predetermined by a window made of sterilized plain paper on the rear dorsal surface of the tongue, was firmly scraped 10 times with sterilized toothpicks. All samples were immediately introduced into an anaerobic chamber containing 80 % N_2, 10 % CO_2 and 10 % H_2 (model AZ-Hard, Hirasawa) and were suspended in 1 ml of distilled 40 mM potassium phosphate buffer (PPB, pH 7·0) solution. After homogenization for 5 min, decimal dilutions from 10^{-3} to 10^{-6} were prepared in 40 mM PPB solution.

Culture conditions. One hundred microlitres from each dilution sample was dispersed and spread either onto Fastidious Anaerobe Agar (FAA, Lab M) plates containing 0·05 % L-cysteine, supplemented with 5 % rabbit blood (Nippon Bio-Test Laboratories), 0·12 % glutathione and 0·02 % lead acetate, according to the method of Paryavi-Gholami *et al.* (1999) with minor modifications, or onto FAA plates without 0·02 % lead acetate as a control. Plates were incubated at 37 °C for 2 weeks in an anaerobic chamber. To ensure strictly anaerobic conditions in the chamber, reduction of methylviologen (−446 mV) was carefully confirmed whenever experiment procedures were carried out.

After 2 weeks of incubation, bacteria forming black or grey colonies were regarded as H_2S-producing. All of the black or grey colonies on plates with less than 100 colonies were picked up using sterilized plastic loops or toothpicks and subcultured on FAA agar plates. These bacterial isolates were confirmed as producing H_2S in test tubes of Fastidious Anaerobe Broth (Lab M) liquid media. Bacterial isolates were grown anaerobically, and the presence of H_2S in the headspace of the test tubes was determined from the blackening of filter paper strips immersed in lead acetate.

DNA extraction and 16S rRNA gene sequencing. Colonies subcultured from four malodourous and four nonodourous subjects were harvested by centrifugation at 7700 ***g*** for 5 min and the supernatant was removed. Genomic DNA was then extracted from the pellets using the InstaGene Matrix Kit (Bio-Rad) according to the manufacturer's instructions.

The 16S rRNA gene sequences were amplified by PCR using universal primers 27F and 1492R (Lane, 1991) and *Taq* DNA polymerase (HotStarTaq Master Mix, Qiagen) according to the manufacturer's instructions. The primer sequences were: 27F, 5′-AGAGTTT GATCMTGGCTCAG-3′; and 1492R, 5′-TACGGYTACCTTGTTAC GACTT-3′. Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed as follows: 15 min at 95 °C for initial heat activation and 35 cycles of 1 min at 94 °C for denaturation, 1 min at 52 °C for annealing and 1·5 min at 72 °C for extension, followed by 10 min at 72 °C for final extension. PCR products were sequenced at Hokkaido System Science using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100, Applied Biosystem). Primers 27F and 1492R were used to sequence both strands (at least 1000 bp), and DNA data were analysed using the DNASIS program (Hitachi Software Engineering). BLAST searches were performed through the website of the National Center for Biotechnology Information. Bacterial species were determined by percentage sequence similarity (>97 %).

Data analysis. An unpaired *t*-test was used to analyse significance. *P* values of $< 0{\cdot}05$ were considered statistically significant.

RESULTS AND DISCUSSION

Relationship between oral malodour level and clinical indicators

Based on the results of GC, the 10 subjects were divided into the H_2S-undetected group (below the detection limit) and the H_2S-detected group (mean ± SD, 1·05 ± 0·97 p.p.m.) (Table 1). In addition, there were significant differences between the two groups in Breathtron and organoleptic scores, which were also used to assess oral malodour (Table 1). Therefore, the H_2S-detected group was designated the odour group, and the H_2S-undetected group was designated the no/low odour group. Methyl mercaptan was detected only in two subjects belonging to the odour group and no dimethyl sulfide was detected.

With regard to clinical parameters, there were no significant differences in age, number of present teeth, number of teeth with untreated caries, number of teeth with probing depth >4 mm, largest probing depth or O'Leary plaque control record score between the two groups (Table 1). There were also no significant differences in tongue coating score between the two groups (Table 1). Considering that the maximum observable tongue coating score is 9, the mean

Table 1. Clinical assessment of no/low odour and odour groups in this study

Data are presented as mean ± SD.

Assessment	No/low odour group ($n = 5$)	Odour group ($n = 5$)
Age	41·8 ± 13·9	30·8 ± 2·4
No. of teeth present	24·0 ± 3·5	27·2 ± 1·3
No. of teeth with untreated caries	0·2 ± 0·48	0·2 ± 0·49
No. of teeth with probing depth >4mm	1·6 ± 2·5	1·6 ± 3·6
Largest probing depth (mm)	3·8 ± 0·8	4·0 ± 2·2
Plaque control record score	38·9 ± 22·9	47·5 ± 23·2
Tongue coating score	1·2 ± 1·1	2·0 ± 1·2
H_2S concentration* (p.p.m.)	Not detected†	1·05 ± 0·97‡
Breathtron score (p.p.b.)	41·50 ± 17·76	1129 ± 903‡
Organoleptic score	0·30 ± 0·30	1·29 ± 0·40‡

*Determined by gas chromatography.

†Below the detection limit.

‡Significantly different ($P < 0·05$) from the no/low odour group.

scores in this study were relatively low (1·2 and 2·0 in the no/low odour and odour groups, respectively; Table 1). In addition, there were no significant differences in thickness score of observable tongue coating between the two groups (data not shown).

Relationship between oral malodour level and densities of total bacteria and H_2S-producing bacteria in tongue biofilm

After 2 weeks of anaerobic incubation, black or grey colonies were observed on plates containing lead acetate and these were designated H_2S-producing bacteria. Few black or grey colonies appeared on plates when the same samples were cultured without lead acetate (data not shown). Total numbers of colonies on plates with and without lead acetate were almost equal, thus indicating that lead acetate did not inhibit bacterial growth. Black or grey isolates were subcultured and confirmed to produce H_2S.

The total number of bacteria (total c.f.u.) in the odour group (mean, $1·4 \times 10^8$) was significantly higher than that in the no/low odour group (mean, $1·3 \times 10^7$; $P < 0·005$) (Fig. 1). This is consistent with previous studies by Hartley *et al.* (1996, 1999). In addition, the number of black or grey colonies in the odour group (mean, $6·4 \times 10^7$) was significantly higher (approximately six-fold) than that in the no/low odour group (mean, $8·1 \times 10^6$; $P < 0·05$). This suggests that H_2S-producing bacteria in the tongue biofilm are the source of oral malodour.

On the other hand, there was no significant difference in the percentage of black or grey colony-forming units among the total colony-forming units between the two groups, although this percentage varied among individuals (20–89 %) (Fig. 2). In this study, tongue biofilm samples were obtained from the same part of the tongue using a standardized method, and no significant differences were noted in observable tongue coating and thickness scores between the two groups (Table 1). This indicates that the amounts of observable tongue coating were similar among the subjects in this study. These results suggest that the number of bacteria per unit of tongue

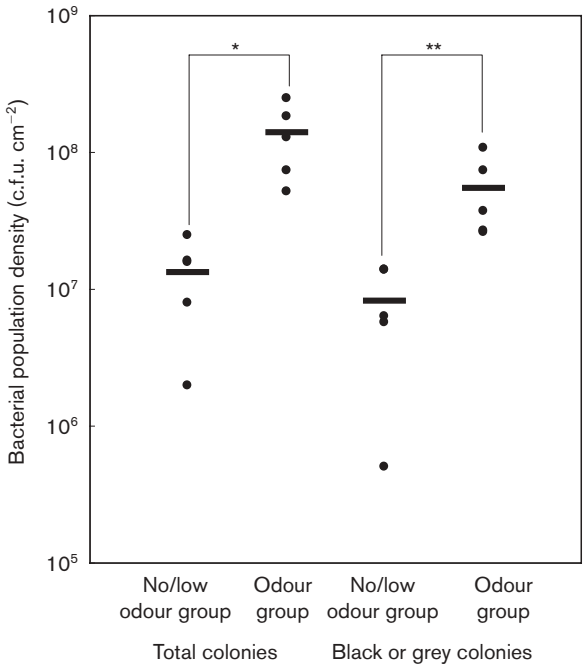

Fig. 1. Numbers of total and black or grey colonies in no/low odour and odour groups. *$P = 0·002$; **$P = 0·012$. Horizontal bars represent means.

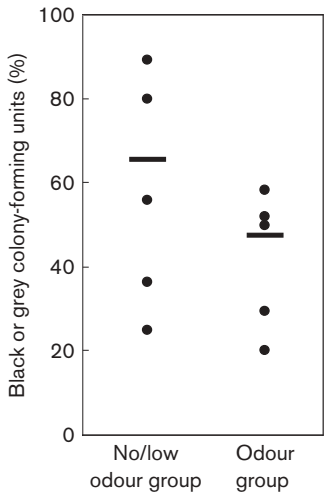

Fig. 2. Proportion of black or grey colony-forming units among total colony-forming units in no/low odour and odour groups. Horizontal bars represent means.

biofilm, i.e. bacterial density in the tongue biofilm, is higher in subjects with oral malodour than in those without oral malodour, and that H_2S-producing bacteria in tongue biofilm are responsible for oral malodour.

Yaegaki & Sanada (1992a, b) and Miyazaki *et al.* (1995) reported correlations between the degree of oral malodour, the amount of observable tongue coating and/or periodontal conditions. In addition, it is suggested that periodontal disease can induce observable tongue coating accumulation (Yaegaki & Sunada, 1992a). The tongue biofilm comprises not only micro-organisms but also epithelial cells released from the oral mucosa and leukocytes from periodontal pockets. Salivary levels of the latter two components could be elevated in patients with periodontal disease, thus leading to an increase in the amount of observable tongue coating. This indicates that the amounts of observable tongue coating bear little relationship to the microbial population density on the tongue coating and it is only the latter (microbial density) that relates to hydrogen sulfide levels or oral malodour.

Hartley *et al.* (1996) reported that the percentage of H_2S-producing bacteria in subjects with strong oral malodour (organoleptic scores >3 on a 0–5 scale) was higher than that in the no/low odour group. In our study, however, a significant correlation was observed with the number rather than the percentage of H_2S-producing bacteria. Organoleptic scores of the subjects with oral malodour in our study were lower (mean 1·29 on a 0–4 scale) (Table 1) than those in the study by Hartley *et al.* (1996) (mean 3·84 on a 0–5 scale). The discrepancy could thus be explained as follows: oral malodour increases with the number of both total and H_2S-producing bacteria in the tongue biofilm, and then becomes more severe as the percentage of H_2S-producing bacteria increases.

Identification of H_2S-producing bacteria in tongue biofilm

The H_2S-producing bacteria isolated in this study were identified using molecular biological methods. *Veillonella*, *Actinomyces* and *Prevotella* species were the predominant H_2S-producing bacteria, followed by *Streptococcus* species, in the odour and no/low odour groups (Table 2). *Veillonella dispar* accounted for over 15 % of total H_2S-producing bacteria in each sample. However, there were no significant differences in the profiles of H_2S-producing bacteria between the two groups.

Hartley *et al.* (1996) also frequently identified these bacterial species in both odour and no/low odour groups, and Donaldson *et al.* (2005) reported that *Veillonella*, *Prevotella* and *Fusobacterium* species were found in both odour and no/low odour groups, and that *Vibrio* species and unidentifiable Gram-negative and Gram-positive anaerobes were more commonly found in the odour group. Loesche & Kazor (2002) reported that 74 % of total cultivable bacteria of the tongue biofilm could be *Veillonella parvula*, *Actinomyces odontolyticus*, *Streptococcus intermedius* and *Clostridium innocuum*, and Mager *et al.* (2003) reported that a *Veillonella* species was one of the prominent bacteria in the tongue biofilm. However, in all these studies, the H_2S-productivity of the bacteria was not assessed. Thus, our study is the first report to show that *Veillonella*, *Actinomyces* and *Prevotella* are predominant as H_2S-producing bacteria in tongue biofilm and are responsible for oral malodour when they increase in number.

Actinomyces species are saccharolytic bacteria that produce lactic acids from carbohydrates, while *Veillonella* species utilize lactic acids as a carbon and energy source instead of carbohydrates. In a mixed culture where carbohydrate is supplied, *Veillonella* species are able to grow together with *Actinomyces* species (Distler & Kröncke, 1981), indicating that *Actinomyces* supply lactic acids to *Veillonella* species. This suggests that, in the tongue coating, *Actinomyces* and *Veillonella* species create a food chain and subsequently establish a stable microbial ecosystem.

In the tongue coating, cysteine and proteins/peptides containing cysteine are thought to be supplied by saliva and desquamated tongue epithelia, and are degraded into H_2S through bacterial metabolism. Some isolates of *Actinomyces* and *Veillonella* have been reported to produce H_2S during growth (Persson *et al.*, 1990; Schaal, 1986; Shibuya, 2001), as shown in this study (Table 2). This indicates that members of *Actinomyces* and *Veillonella* possess an enzyme responsible for the breakdown of cysteine into H_2S, although no information is available regarding cysteine-degrading enzymes such as cysteine desulfhydrase (Claesson *et al.*, 1990; Pianotti *et al.*, 1986) in these bacteria. *Prevotella* species including *Prevotella veroralis* ferment amino acids and some

Table 2. H_2S-producing bacterial species in tongue biofilm of eight subjects

Species	No. (%)* isolated from each subject									
	No/low odour group					Odour group				
	1	2	3	4	Total	5	6	7	8	Total
Total H_2S-producing isolates	58 (50·9)†	63 (39·4)†	64 (80·0)†	57 (72·2)†	242 (55·9)†	10 (55·5)†	24 (46·2)†	39 (52·7)†	53 (42·4)†	126 (46·8)†
Gram-positive cocci										
Atopobium parvulum	1 (1·7)	0 (0·0)	1 (1·6)	4 (7·0)	6 (2·5)	0 (0·0)	0 (0·0)	0 (0·0)	4 (7·5)	4 (3·2)
Gemella sanguinis	1 (1·7)	0 (0·0)	0 (0·0)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Micromonas micros	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Streptococcus mitis	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Streptococcus parasanguinis	0 (0·0)	3 (4·8)	0 (0·0)	0 (0·0)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Streptococcus vestibularis	0 (0·0)	11 (17·5)	2 (3·1)	0 (0·0)	13 (5·4)	0 (0·0)	0 (0·0)	0 (0·0)	8 (15·1)	8 (6·3)
Gram-positive rods										
Actinomyces graevenitzii	0 (0·0)	0 (0·0)	0 (0·0)	3 (5·3)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	1 (1·9)	1 (0·8)
Actinomyces odontolyticus	0 (0·0)	8 (12·7)	32 (50·0)	0 (0·0)	40 (16·5)	2 (20·0)	5 (20·8)	13 (33·3)	11 (20·8)	31 (24·6)
Eubacterium saburreum	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Eubacterium species	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Mogibacterium species	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	1 (2·6)	0 (0·0)	1 (0·8)
Gram-negative cocci										
Megasphaera micronuciformis	1 (1·7)	4 (6·3)	0 (0·0)	1 (1·8)	6 (2·5)	0 (0·0)	0 (0·0)	4 (10·3)	2 (3·8)	6 (4·8)
Veillonella dispar	27 (46·6)	26 (41·3)	10 (15·6)	48 (84·2)	111 (45·9)	8 (80·0)	6 (25·0)	16 (41·0)	16 (30·2)	46 (36·5)
Veillonella parvula	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	2 (8·3)	0 (0·0)	0 (0·0)	2 (1·6)
Veillonella species	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Gram-negative rods										
Bulleidia moorei	1 (1·7)	3 (4·8)	2 (3·1)	0 (0·0)	6 (2·5)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Campylobacter concisus	1 (1·7)	1 (1·6)	0 (0·0)	0 (0·0)	2 (0·8)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Campylobacter showae	6 (10·3)	0 (0·0)	0 (0·0)	0 (0·0)	6 (2·5)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Capnocytophaga gingivalis	3 (5·2)	0 (0·0)	0 (0·0)	0 (0·0)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Fusobacterium nucleatum	0 (0·0)	0 (0·0)	6 (9·4)	0 (0·0)	6 (2·5)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Prevotella veroralis	10 (17·2)	5 (7·9)	0 (0·0)	0 (0·0)	15 (6·2)	0 (0·0)	0 (0·0)	4 (10·3)	7 (13·2)	11 (8·7)
Prevotella melaninogenica	1 (1·7)	0 (0·0)	1 (1·6)	0 (0·0)	2 (0·8)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Prevotella species	0 (0·0)	0 (0·0)	2 (3·1)	0 (0·0)	2 (0·8)	0 (0·0)	2 (8·3)	0 (0·0)	0 (0·0)	2 (1·6)
Selenomonas dianae	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Selenomonas species	1 (1·7)	0 (0·0)	0 (0·0)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Unidentified	5 (8·6)	2 (3·2)	3 (4·7)	1 (1·8)	11 (4·5)	0 (0·0)	5 (20·8)	1 (2·6)	4 (7·5)	10 (7·9)

*Percentage among H_2S-producing isolates from each subject except where indicated.

†Percentage among total bacterial isolates from each subject.

species possess proteolytic activity (Shah & Collins, 1990), thus suggesting that these species can degrade proteins/peptides and ferment the resultant cysteine into H_2S as detected in our study.

Periodontal disease-associated bacteria such as *Porphyromonas gingivalis* and *Prevotella intermedia*, which produce VSCs (Loesche & Kazor, 2002; Persson *et al.*, 1990), were not detected in the present study (Table 2), in which no periodontal disease patients were included (Table 1). *Fusobacterium* species, known to be VSC-producing periodontal inhabitants (Claesson *et al.*, 1990), were scarcely detected (Table 2). These results suggest that periodontal disease-associated bacteria are not associated with oral malodour in patients without periodontal disease or with low to intermediate levels of oral malodour.

Conclusions

H_2S-producing bacteria in the tongue biofilm appear to cause low to intermediate levels of oral malodour in patients without periodontitis, and the predominant H_2S-producing bacteria are mainly commensal species of the oral cavity, such as *Veillonella* and *Actinomyces* species. Furthermore, the numbers of both H_2S-producing bacteria and total bacteria in the tongue biofilm were higher in the odour group, suggesting that for subjects with low to intermediate levels of malodour an increase in bacterial density in the tongue biofilm is associated with oral malodour.

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