Identification of oral bacterial species associated with halitosis

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Oral halitosis—bad breath originating from the oral cavity—regularly affects about one in four adults[1] and frequently is caused by bacteria infecting the dorsal surface of the tongue and producing volatile sulfur compounds (VSCs).[2] (Throughout the article, we use the term halitosis when referring to oral halitosis.) Until recently, most studies of infectious diseases focused on microorganisms identified by traditional bacterial culture methods. Newer methods, however, such as the direct amplification of microbial nucleic acids (also called broad-range polymerase chain reaction [PCR]),[3] can identify both cultivable and noncultivable microorganisms. This is important because noncultivable bacterial species are more numerous than cultivable bacterial species.[4] Consequently, new microorganisms are being discovered by direct amplification of microbial nucleic acids, and these techniques have doubled the number of bacterial species estimated to infect the human oral cavity from 400 to 800.[5] The most tangible result of these new methods is the identification of pre-

ABSTRACT

Background. The authors examined the tongue bacteria associated with oral halitosis (bad breath originating from the oral cavity), focusing on noncultivable bacteria—bacteria that cannot be identified by bacterial culture techniques.

Methods. The authors took samples from the dorsal tongue surface of eight adult subjects with halitosis and five control subjects who did not have halitosis. They identified the bacteria in these samples by using both anaerobic culture and direct amplification of 16S ribosomal DNA, a method that can identify both cultivable and noncultivable microorganisms. They analyzed the resulting microbiological data using χ^2 and correlation coefficient tests.

Results. Clinical measures of halitosis were correlated highly with each other and with tongue coating scores. Of 4,088 isolates and phylotypes identified from the 13 subjects, 32 species including 13 noncultivable species were found only in subjects with halitosis. *Solobacterium moorei* was present in all subjects with halitosis but not in any control subjects.

Conclusions. Subjects with halitosis harbor some bacterial species on their dorsal tongue surfaces that are distinct from bacterial species found in control subjects. This finding is consistent with the hypothesis that halitosis has a microbial etiology.

Clinical Implications. Like other oral diseases with microbial etiology, halitosis may be amenable to specific and nonspecific antimicrobial therapy targeted toward the bacteria associated with it.

Key Words. Halitosis; *Solobacterium moorei;* direct amplification; polymerase chain reaction; anaerobic culture.

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viously unknown microbial etiologies for infectious diseases such as *Tropheryma whippleii* in Whipple's disease[6] and *Prevotella bergensis* in skin infections.[7]

In this study, we used direct amplification of microbial nucleic acids together with traditional bacterial culture to identify the bacteria infecting the dorsal surface of the tongue in subjects with halitosis.

SUBJECTS, MATERIALS AND METHODS

Subjects. Thirteen adults (eight men, five women; mean age 41 years, range 21-71 years) participated in the study. Eight of the subjects had halitosis (six men, two women; mean age 47 years, range 21-71 years), and five subjects did not have halitosis (two men; three women; mean age 33 years, range 21-55 years). We explained the study to the subjects, who acknowledged their agreement by signing an informed consent statement. Each subject had at least 20 erupted natural teeth, including at least one molar and one premolar in each quadrant. Subjects did not have systemic disease, did not use a removable partial denture, and had not received antibiotics in the past three months. Two of the subjects smoked. All of the subjects reported brushing their teeth at least once a day, but none reported brushing their tongues. For 12 hours before testing, subjects refrained from undergoing their customary oral hygiene procedures, as well as using oral rinses or breath fresheners.

Halitosis assessment. We determined the presence and severity of halitosis three ways. First, in an organoleptic assessment, we scored the subjects' breath from 0 (no appreciable odor) to 5 (extremely foul odor); we considered a score from 3 to 5 to be positive. Second, we used a portable sulfide detector (Halimeter, Interscan, Chatsworth, Calif.) to determine the sulfur content of the subject's mouth air; we considered greater than 250 parts per billion to be positive.[8] Third, we used a VSC/polyamine assay; we considered "medium" and "high" reactions to be positive. For the purposes of our study, we defined subjects as having oral halitosis only if they were positive for all three assessments. Control subjects were negative for all three assessments. We also determined the thickness and extent of tongue coating (0 = no coating to 3 = heavy coating).[9]

Sampling and bacterial culture. We took samples by gently scraping an area of the dorsal tongue surface that was approximately 2 square centimeters and placed the sample in 3 milliliters of prereduced anaerobically sterilized Ringer's solution.

After dispersing the sample to achieve as uniform a bacterial cell suspension as possible, we determined the number of bacterial cells per milliliter by phase contrast microscopy using a Petroff-Hauser Bacteria Counter (Horsham, Pa.). We determined the number of colony-forming units after anaerobically incubating the samples at 37 C for five to seven days on enriched tryptic soy agar. From each cultured sample, we randomly selected up to 200 bacterial colonies, isolated them to purity and identified them by analyzing the 16S ribosomal DNA (rDNA) sequence as described below.

Direct amplification. We isolated DNA from the original sample and from randomly selected bacterial colonies using a DNA isolation kit. We amplified bacterial 16S rDNA genes by PCR using a thermocycler and primers (5'-AGAGTTTGATCA/CTGG-3' and 5'-TACCTTGTTACGACTT-3'). We performed the amplification for 30 cycles of 30 seconds at 95 C, 30 seconds at 55 C and 30 seconds at 72 C. The negative control samples contained all the PCR reagents except for the sample DNA. The positive control samples contained all the PCR reagents together with *Escherichia coli* DNA. We cloned the PCR-amplified DNA into *E. coli* using a cloning kit. We isolated the DNA from the transformed cells, sequenced the DNA and identified the bacteria by comparing the DNA sequence to published sequences.

Statistical analysis. We used a correlation coefficient test to examine the relationship between clinical indexes. We used a χ^2 analysis to examine the association between bacterial species and halitosis. We considered P values of $< .05$ to be significant.

RESULTS

Table 1 lists the demographic and clinical data for subjects in our study. As might be expected based on the patient selection criteria, we found a strong correlation between the different measures of halitosis: organoleptic scores and VSC ($r = 0.85$; $P = .0003$) and organoleptic scores and

ABBREVIATION KEY. PCR: Polymerase chain reaction. **rDNA:** Ribosomal DNA. **VSC:** Volatile sulfur compounds.

VSC/polyamine assays ($r = 0.92$, $P = .0021$). We also found a strong correlation between the measures of halitosis and the tongue coating scores: organoleptic scores and tongue coating scores ($r = 0.89$; $P = .0021$), VSC and tongue coating scores ($r = 0.74$; $P = .001$), and VSC/polyamine assays and tongue coating scores ($r = 0.91$, $P = .0003$).

We identified 4,088 bacterial isolates and phylotypes (genetic signatures) in the samples we took from the 13 subjects. From the eight subjects with halitosis, we identified 2,768 bacterial isolates and phylotypes, including 1,204 cultivable isolates and 1,564 phylotypes. We identified 84 bacterial species in the group of subjects with halitosis, and each subject had 16 to 23 species. From the five control subjects, we identified 1,320 bacterial isolates and phylotypes, including 580 isolates from cultures and 740 phylotypes identified by direct amplification of 16S rDNA. We identified 69 species in the control group, and each subject had 11 to 19 species.

TABLE 1

Demographic data and clinical assessments of the study population.

GROUP (SUBJECT NUMBER)	AGE (YEARS)	SEX	ORGANOLEPTIC SCORE*	VSC† (PPB‡)	VSC/POLYAMINE ASSAY	TONGUE COATING§
Control						
1	45	F	1	130	Low	0
2	24	M	1	100	Low	0
3	23	F	1	120	Low	0
4	55	M	1	140	Low	0
5	21	F	1	120	Low	0
Halitosis						
1	47	F	5	779	High	3
2	21	M	4	386	High	3
3	70	M	4	432	Medium	2
4	25	M	3	250	Medium	1
5	31	F	4	363	High	2
6	71	M	5	310	High	3
7	51	M	4	280	High	3
8	60	M	4	425	High	3

* 0 = no appreciable odor to 5 = extremely foul odor.
† VSC: Volatile sulfur compound.
‡ PPB: Parts per billion.
§ 0 = no coating to 3 = heavy coating.

Table 2 lists the most prevalent bacterial species in the halitosis and the control groups. *Streptococcus salivarius* was found in all subjects and was the most frequently identified bacterial species. *S. salivarius* comprised 12.87 percent of the 1,320 isolates and phylotypes from the control group (range, 1.3-21.0 percent) and 17.7 percent of the 2,768 isolates and phylotypes from the halitosis group (range, 6.1-38.6 percent).

The next most prevalent species found in all of the control subjects were *Prevotella melaninogenica* (8.95 percent of the total isolates and phylotypes; range, 2.7-13.2 percent), *Streptococcus parasanguinis* (8.32 percent; range, 4.5-23.0 percent), *Campylobacter concisus* (7.77 percent; range, 1.3-17.9 percent) and *Streptococcus mitis* (6.99 percent; 0.7-13.2 percent). Also present in all of the control subjects, but not as prevalent were *Veillonella atypica* (2.59 percent; range, 0.5-8.3 percent), *Streptococcus* species (2.51 percent; range, 0.5-8.4 percent), *Streptococcus sanguinis* (2.12 percent; range, 0.4-4.8 percent), *Fusobacterium nucleatum* (2.04 percent; range, 0.5-3.8 percent) and *Veillonella parvula* (1.49 percent; range, 0.5-3.7 percent).

The next most prevalent species found in all of the subjects with halitosis were *S. parasanguinis* (12.91 percent of the total isolates and phylotypes from the halitosis group; range, 1.4-18.3 percent) and *Actinomyces odontolyticus* (8.51 percent; range, 0.8-19.5 percent). Also present in all of the subjects with halitosis were *P. melaninogenica* (7.33 percent; range, 1.8-20.8 percent), *S. mitis* (5.76 percent; range, 1.4-16.0 percent), *Solobacterium moorei* (4.79 percent; range, 0.9-10.8 percent), *Streptococcus oralis* (3.50 percent; range, 0.6-13.3 percent), *Granulicatella adiacens* (2.00 percent; range, 0.2-5.8 percent) and *S. sanguinis* (1.07 percent; range, 0.2-2.7 percent).

We identified 51 percent of the species in both the halitosis and control groups. For example, we

TABLE 2

Most prevalent bacterial species on the human tongue.*

BACTERIAL SPECIES	CONTROL GROUP						
	1	2	3	4	5	% Isolates†	% Prevalence
Streptococcus salivarius	4.3§	21.0	13.2	17.9	1.3	12.87	100
Prevotella melaninogenica	2.7	8.6	13.2	10.7	8.4	8.95	100
Streptococcus parasanguinis	23.0	4.9	6.6	4.5	8.0	8.32	100
Campylobacter concisus	4.3	5.8	17.9	10.3	1.3	7.77	100
Streptococcus mitis	5.9	13.2	6.6	0.7	6.8	6.99	100
Actinomyces odontolyticus	11.2	0.0	3.8	9.3	4.2	5.18	80
Prevotella species	0.5	1.7	0.0	5.9	14.8	4.63	80
Actinomyces meyeri	9.1	1.7	4.7	0.0	3.4	3.22	80
Streptococcus oralis	4.3	4.6	2.8	0.0	4.2	3.14	80
Streptococcus infantis	3.7	8.3	0.0	0.3	0.4	2.98	80
Veillonella atypica	0.5	1.4	0.5	8.3	0.8	2.59	100
Streptococcus species	0.5	1.4	1.9	0.7	8.4	2.51	100
Veillonella dispar	1.6	0.9	0.0	6.6	1.3	2.20	80
Granulicatella adiacens	8.0	2.3	0.5	0.0	1.3	2.12	80
Streptococcus sanguinis	4.8	0.9	1.4	3.8	0.4	2.12	100
Fusobacterium nucleatum	0.5	1.4	3.8	2.1	2.5	2.04	100
Prevotella veroralis	0.0	2.0	0.9	3.5	0.0	1.49	60
Veillonella parvula	3.7	1.2	0.5	1.0	1.7	1.49	100
Rhodococcus opacus	0.5	1.7	0.0	0.0	1.3	0.78	60
Prevotella pallens	0.0	0.0	0.5	0.0	3.0	0.63	40
Gemella species oral strains	3.7	0.0	0.0	0.0	0.0	0.55	20
Actinomyces species	0.5	0.0	0.0	1.7	0.0	0.47	40
Actinomyces graevenitzii	1.1	0.0	0.0	0.0	0.0	0.16	20
Solobacterium moorei	0.0	0.0	0.0	0.0	0.0	0.00	0
Haemophilus paraphrophilus	0.0	0.0	0.0	0.0	0.0	0.00	0

Continued

isolated *S. salivarius* from each subject, and it composed a significant proportion of the microflora in both groups. We identified some species in both groups, but they represented a significant proportion of the total microflora in only one group, suggesting that they were associated more closely with that group. For example, we identified *S. parasanguinis* in each subject, but it constituted a much larger proportion of the microflora (> 10 percent) in the subjects with halitosis.

There was greater bacterial diversity in samples taken from the subjects with halitosis. We found 32 species (including 13 "uncultured" or "unidentified" species) only in the halitosis group, while we found 17 species only in the control group. *S. moorei* was key among the species unique to the halitosis group (Table 3, page 1118). We identified it in each of the subjects with halitosis in proportions as high as 10.8 percent, and we found that it was associated significantly with halitosis $\chi^2 = 0.22$). This bacterium constituted 4.79 percent of the 2,768 bacterial isolates and phylotypes from the halitosis samples and was the seventh most numerous species identified in the subjects with halitosis.

We found a number of species only in the sam-

TABLE 2 (CONTINUED)

HALITOSIS GROUP									
1	2	3	4	5	6	7	8	% Isolates‡	% Prevalence
23.1	38.6	6.1	15.8	15.9	11.8	21.5	13.5	17.7	100
1.8	6.0	2.7	10.8	3.8	2.5	7.3	20.8	7.33	100
17.6	15.5	1.4	8.2	18.3	10.7	12.2	18.3	12.91	100
1.3	0.0	1.4	1.4	4.1	4.9	2.0	1.3	2.11	88
3.7	9.6	16.0	3.3	1.4	3.8	6.1	5.6	5.76	100
1.6	0.8	4.1	8.5	15.1	19.5	13.7	3.0	8.51	100
0.0	2.8	0.0	2.6	1.2	0.8	0.0	0.3	.093	63
0.0	2.0	2.0	13.4	2.3	0.8	7.6	2.3	4.08	88
2.6	2.4	13.3	3.1	0.6	2.5	1.2	3.8	3.50	100
0.3	0.0	0.0	0.0	1.2	6.3	0.6	4.6	1.72	63
6.8	0.8	0.0	1.4	1.4	4.4	1.2	7.6	3.18	88
3.4	2.8	19.5	0.2	0.3	0.0	0.3	1.0	3.00	88
8.1	1.6	0.0	2.1	2.0	4.1	1.2	0.3	2.54	88
2.1	1.2	5.8	0.2	2.0	1.4	1.7	2.3	2.00	100
0.8	0.8	1.4	0.2	0.6	2.7	1.7	0.5	1.07	100
0.0	0.4	2.7	0.5	0.9	0.8	1.2	0.0	0.75	75
0.5	2.4	0.0	3.5	0.6	0.0	0.9	1.3	1.18	75
3.1	0.8	1.0	0.2	2.0	0.0	0.3	0.5	1.00	88
0.0	0.0	0.0	0.0	0.0	0.3	6.7	0.0	0.86	25
0.0	0.8	0.3	1.9	0.3	1.1	0.0	1.8	0.82	75
0.0	0.0	4.1	0.2	0.9	1.4	0.6	0.0	8.22	63
1.0	0.0	0.3	2.8	0.9	1.4	1.2	1.0	1.18	75
0.0	0.8	0.3	0.2	3.5	0.5	0.6	0.3	0.75	88
10.0	1.2	1.0	10.8	8.4	1.9	0.9	1.3	4.79	100
0.0	0.0	0.0	0.0	7.0	0.0	0.0	0.5	0.93	25

* Listed in order of the most prevalent species (percentage isolates) in the samples from the control subjects.
† Percentage of isolates identified by either culture or direct amplification methods from the control subject samples.
‡ Percentage of isolates identified by either culture or direct amplification methods from the subjects with halitosis samples.
§ Percentage of isolates identified by either culture or direct amplification methods from individual subject samples.

ples from the subjects with halitosis, but in lower prevalence and proportions. They include *Abiotrophia defectiva, Atopobium vaginae, Bacteroides caccae, Bacteroides stercoris, Catonella* species, *Clavibacter xyli, Dialister pneumosintes, Enterococcus faecalis, Enterococcus malodoratus, Eubacterium nodatum, Haemophilus parahprophilus, Kingella denitrificans, Luteococcus japonicus, Mobiluncus curtisii, Mogibacterium neglectum, Prevotella oris, Saccharothrix australiensis, Selenomonas infelix, Staphylococcus haemolyticus, Stomatococcus mucilaginosus, Treponema* species and *Veillonella* "oral clone."

We categorized the sequences of 38 isolates (5.3 percent of the 704 phylotypes from the halitosis samples) into 13 "uncultured" or "unidentified" species (Table 4, page 1119). We found none of these bacteria in the control subjects. Included among these bacteria were those in the phylum TM7 (a proposed taxonomic division that includes

TABLE 3

Bacterial species identified only in the control group or the halitosis group.*

BACTERIAL SPECIES	% ISOLATES†	% PREVALENCE
Identified Only in Control Group		
Porphyromonas catoniae	2.0	60
Gemella sanguinis	0.5	40
Identified Only in Halitosis Group		
Solobacterium moorei	4.8	100
Granulicatella elegens	0.6	63
***Eubacterium* species**	0.3	50
***Firmicutes* species**	0.1	50
Unidentified oral bacterium	0.8	38
***Porphyromonas* species**	0.3	38
Staphylococcus warneri	0.1	38
***Dialister* species**	0.5	25
Prevotella intermedia	0.2	25

* Species identified in two or more subjects in the group and in 1 percent or greater of a subject's isolates.
† Percentage of isolates identified by either culture or direct amplification methods from the subject samples.

bacteria that have never been cultured but have been identified only through nucleic acid sequencing) that have been identified in the gingival sulcus[10] and have been associated with chronic periodontitis and necrotizing ulcerative periodontitis.[11]

Species with low prevalence and numbers that we found only in the control group included *Acinetobacter lwoffii, Actinobacillus* species, *Capnocytophaga gingivalis, Capnocytophaga sputigena, Clostridium xylanolyticum, Corynebacterium durum, Enterococcus pallens, Gemella sanguinis, Helicobacter pylori, Kocuria varians, Oribacterium sinus, Porphyromonas catoniae, Rothia aeria* and *Streptomyces* species.

We detected several species only by direct amplification of nucleic acids. They included *Abiotrophia defectiva, Firmicutes* species, *Dialister* species, *Selenomonas* species, *Leptotrichia* species, *Catonella* species, *Luteococcus* species and *Corynebacterium durum*. We rarely isolated several other species by culture, but we could detect them readily using direct amplification of 16S rDNA. They included *Atopobium parvulum, Enterococcus* species, *Granulicatella* species, *Neisseria* species and *S. moorei*.

DISCUSSION

Although the microbiology of the human oral cavity has been investigated thoroughly (there are more than 22,000 resulting PubMed citations when the search term "oral microbiology" is used), studies that used direct amplification of 16S rDNA (broad-range PCR) indicated that the oral microflora remained incompletely characterized.[3-5] Most previous studies focused on cultivable microorganisms, which constituted only 1 to 10 percent of all microbial species. Consequently, previous studies have been biased toward "what grows" and have ignored "what does not grow": the noncultivable species. As opposed to bacterial culture, direct amplification of 16S rDNA can identify both cultivable and noncultivable bacteria. This method has identified previously unidentified infectious agents such as *T. whippleii* in Whipple's disease[6] and *P. bergensis* in skin infections,[7] as well as infectious agents in "culture negative" clinical samples.[12]

Using direct amplification of 16S rDNA, we identified 38 phylotypes that we categorized into 13 "uncultured" or "unidentified" species based on their nuclei acid sequences. This is consistent with previous reports indicating that direct amplification of 16S rDNA can identify greater numbers of oral bacteria than can culture.[13] Among the bacteria we identified only by direct amplification were those in the phylum TM7, which has been associated with periodontal disease,[10] and several species normally associated with the urogenital tract.

The results of both the bacterial culture assays and the direct amplification assays confirm the importance of oral microorganisms in halitosis and point to differences in dorsal tongue bacteria between subjects with halitosis and those without. *Veillonella* species, *Actinomyces* species and *Streptococcus* species were the bacteria predominantly found in both control subjects and subjects with halitosis. Similar to a study by Kazor and colleagues,[5] we identified *S. salivarius* most frequently in our study. Among species we identified in two or more subjects in the group and in proportions of 1 percent or more of a subject's isolates, we identified two species exclu-

sively in healthy subjects, while we identified nine exclusively in subjects with halitosis. This suggests that there is greater microbial diversity in the subjects with halitosis, as was previously reported by Donaldson and colleagues.[14] Two patients with halitosis in our study had mild-to-moderate generalized chronic periodontitis and harbored periodontal pathogens including *Prevotella intermedia* and *Fusobacterium* species, which both produce VSCs.[15,16] However, the remaining subjects with halitosis in our study did not have periodontal disease or identifiable periodontal pathogens, which implicated other species as causing their halitosis. Washio and colleagues[17] made similar observations.

Prominent among the species associated with halitosis in our study was *S. moorei,* a gram-positive bacterium originally isolated from human feces[18] that has been associated with bacteremia,[19] septicemia[20] and refractory cases of endodontic infections.[21] In our study, we found *S. moorei* in all subjects with halitosis, but we did not detect it by either culture or direct amplification of 16S rDNA in any of the control subjects. Kazor and colleagues[5] also associated halitosis with *S. moorei,* identifying it in one of five subjects without halitosis and in three of six subjects with halitosis. A recent study showed that a number of antimicrobial agents and antibiotics are effective in vitro against *S. moorei* and, presumably, against other bacteria associated with halitosis, leading to the possibility of antimicrobial therapy for halitosis.[22] However, the clinical efficacy of such specific and nonspecific agents in treating halitosis is still to be determined.

As previously observed,[2,23,24] we found a strong correlation between a thick yellow or gray tongue coating and halitosis. Tongue coatings are comprised of desquamated epithelial cells, blood cells and bacteria. The dorsal tongue surface is prone to bacterial accumulation. More than 100 bacteria can attach to a single epithelial cell on the dorsal tongue compared with about 25 bacteria that can attach to other types of oral epithelial cells. Furthermore, structural features of the tongue such as tongue fissures or crenations can create a low-oxygen microenvironment that is protected from the flushing action of the saliva and favors the growth of anaerobic bacteria that may produce VSCs.

Although halitosis generally is considered to be an esthetic problem, it may have implications for systemic health. There is evidence to suggest that even low concentrations of VSCs may be toxic.[25] Toxicity may be related to the mechanisms of action of the agents that comprise VSCs. For example, hydrogen sulfide can split protein disulfide bonds to form persulfide groups; bind metal ions[26]; inhibit enzymes such as myeloperoxidase,[27] catalase,[27] carbonic anhydrase and sodium/potassium adenosine triphosphatase; and potentiate the mutagenicity of hydrogen peroxide.[28] Methyl mercaptan, another source of VSCs, increases the permeability of intact mucosa and stimulates cytokine production.[25,29,30] Increased VSC levels also may play a role in the link between oral infection and systemic diseases such as heart disease and preterm low birth weight.[29]

TABLE 4

Phylotypes from subjects with halitosis categorized as 'uncultured' or 'unidentified' species.*

SPECIES	% PREVALENCE
AP0-8 Unidentified Oral Bacteria	13
AP60-1 Unidentified Bacteria	25
AP60-48 Unidentified Bacteria	13
AP60-8 Unidentified Bacteria	13
D0F5A Uncultured Bacteria	13
HstpL15 Uncultured Eubacterium	13
OS9E Uncultured Bacteria	50
OSs78 Uncultured Bacteria	25
PR55-19 Unidentified Bacteria	13
PR55-2 Unidentified Bacteria	13
RP55-19 Unidentified Oral Bacterium	25
RP55-4 Unidentified Oral Bacterium	25
Sp-7 Uncultured Veillonella	13

* The number and letter combinations before the species types are designations that indicate that one organism is not exactly the same as the others.

CONCLUSIONS

Our study suggests that subjects with halitosis are infected with specific species such as *S. moorei* that are not found in subjects without hal-

itosis. Many of the halitosis-associated bacteria produce high levels of VSCs, which are toxic to tissues and may play a role in the pathogenesis of inflammatory conditions. Consequently, halitosis should not be treated simply as an esthetic problem. It has been shown that halitosis is associated with specific bacteria; therefore, it may be amenable to specific and nonspecific antimicrobial therapies. ■

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