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Volatile sulfur compounds in periodontal pockets

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INTRODUCTION

A first line of defense against bacterial infection is constituted by the epithelial cells, which cover our body surfaces. In the oral cavity this contiguous epithelial layer is breached by the teeth, creating sites vulnerable to bacterial insult. The supporting tissues of the teeth are consequently the site of a widespread infectious disease – the periodontal disease. Due to bacterial colonization of the root surfaces of the teeth, proliferation of the juxtaposed epithelium is initiated, and periodontal pockets develop between the root surfaces and the epithelium. When the disease process continues, the collagenous attachment of the teeth to the bone is damaged as well as the bone itself. This leads to loosening and eventual loss of the teeth.

The periodontal pockets become colonized by a very complex bacterial community. The maintenance and the integrity of the tooth supporting tissues is dependent on the efficiency of the host defenses against the bacteria. The epithelium of the pockets provides a mechanical barrier to bacterial invasion. Another barrier is provided by polymorphonuclear leukocytes, which pass from the microcirculation through the epithelium into the pockets. The polymorphonuclear leukocytes effectively keep the bacteria under control by release of antibacterial products into the pocket and by phagocytosis followed by killing. Bacterial activities in the pockets may, however, in various ways perturb the host defense.

As a result of the metabolic activities of the bacteria in the periodontal pockets, volatile sulfur compounds are formed. These compounds are considered to be toxic. In the present work the formation of volatile sulfur compounds by oral bacteria was studied and the possible effects of these compounds on host defense were evaluated.

Sulfur in nature

Sulfur is among the ten most abundant elements on Earth (Jørgensen, 1983). When life appeared on our planet about 3.5 billion years ago, the atmosphere was devoid of oxygen (Gilbert, 1981). The bacteria, representing the first life form, were anaerobic and in their metabolism they probably used organic material formed in the atmosphere and washed into the seas. Later in the evolutionary process organisms began to exploit sunlight, carbon dioxide and hydrogen sulfide in an early form of photosynthesis. Carbohydrates were formed with a concomitant release of elemental sulfur into the oxygen-free environment.

$$CO_2 + 2\,H_2S + \text{sunlight} \rightarrow (CH_2O) + 2\,S^0 + H_2O$$

When the supply of hydrogen sulfide was depleted, water was utilized instead in the carbon fixation by photosynthesis.

$$CO_2 + H_2O + \text{sunlight} \rightarrow (CH_2O) + O_2$$

Oxygen formed in the photosynthesis reacted with ferrous ions in the seas. When this iron was depleted 2 billions years ago, the oxygen level of the atmosphere started to increase and reached its present level around 1 billions years ago (Gilbert, 1981).

Hydrogen sulfide is still important today for some forms of life. Underwater hot springs and many hot springs on land are rich in hydrogen sulfide and support rich microbial communities. In these sites microorganisms obtain energy for carbon fixation from the oxidation of hydrogen sulfide and other reduced forms of sulfur (Childress et al., 1989).

Sulfur exists in multiple oxidation states in nature, ranging from +6 in sulfuric acid and its derivatives to −2 in volatile hydrogen sulfide and its derivatives. Reduction of inorganic sulfate to inorganic or organic sulfide and the subsequent oxidation of sulfide back to sulfate is known as the biological sulfur cycle. Most sulfur on the Earth's surface is in the form of sulfate (Charlson et al., 1987). Sulfate can be used by bacteria in two major pathways. A dissimilatory pathway is present in specialized sulfur bacteria found in anaerobic environments. These bacteria use the sulfate ion in place of oxygen as a terminal electron acceptor in their energy metabolism (Anderson et al., 1992). Sulfate is then reduced to hydrogen sulfide. An assimilatory pathway is found in all plants and many bacteria. Sulfate is reduced and sulfur is built into molecules like the amino acids cysteine and methionine (Anderson et al., 1992). When such organic sulfur compounds are degraded by bacteria, sulfur is usually liberated in the form of hydrogen sulfide, but other volatile sulfur compounds like methyl mercaptan, dimethyl sulfide and dimethyl disulfide, may also be formed.

Volatile sulfur compounds in the oral cavity

Volatile sulfur compounds contribute to oral malodor (Tonzetich, 1971). Hydrogen sulfide, methyl mercaptan, dimethyl sulfide and dimethyl disulfide have been found in mouth air, and in saliva incubated *in vitro* (Berg et al., 1946, 1947; Larsson & Widmark, 1969; Tonzetich, 1971, 1977; Blanchette et al., 1976; Kaizu et al., 1978). Hydrogen sulfide and methyl mercaptan account for about 90% of the total content of volatile sulfur compounds in mouth air (Tonzetich, 1971). When oral bacteria are incubated *in vitro* in saliva or in saliva supplemented with L-cysteine it is mostly Gram negative bacteria which form volatile sulfur compounds (McNamara et al., 1972; Solis-Gaffar et al., 1979).

The presence of hydrogen sulfide has been demonstrated in infected periodontal pockets. Rizzo (1967) observed hydrogen sulfide in pockets with a depth of 2 to 6 mm, and Morhart and coworkers (1970) in all pockets deeper than 3 mm. Horowitz and Folke (1973) demonstrated the presence of hydrogen sulfide in 6% of clinically healthy sites and in 90% of periodontal pockets with a depth of 4 mm or more. In all these studies hydrogen sulfide was demonstrated by the blackening of lead acetate paper inserted into the pockets. However, the actual concentration of hydrogen sulfide or other volatile sulfur compounds in periodontal pockets has not been determined.

The amounts of volatile sulfur compounds present in mouth air and in periodontal pockets decrease, when efficient oral hygiene measures are introduced or the periodontal disease is treated (Morris & Read, 1949; Larsson & Widmark, 1969; Tonzetich, 1971, 1978; Horowitz & Folke, 1973; Larsson 1973; Solis-Gaffar et al., 1975; Tonzetich & Ng, 1976; Schmidt & Tarbet, 1978; Rosenberg et al., 1991).

Although it is known that bacterial communities from periodontal pockets form hydrogen sulfide when incubated in human serum (ter Steeg et al., 1987), it is not known whether other volatile sulfur compounds may also be formed. There may be more than 300 bacterial taxa in the oral cavity (Moore, 1987). The capacity to produce volatile sulfur compounds has been studied only in *Fusobacterium nucleatum* (Pianotti et al., 1986), *Porphyromonas gingivalis, Prevotella intermedia* (Tonzetich & McBride, 1981), and *Veillonella alcalescens* (Rogosa & Bishop, 1964).

Cysteine is the most important source for the production of hydrogen sulfide (Tonzetich & Carpenter, 1971; Tonzetich et al., 1979; Solis-Gaffar et al., 1979, Tonzetich & McBride, 1981; Pianotti et al., 1986) and methionine for methyl mercaptan (Soda et al., 1983; Kiene & Visscher, 1987). Cysteine desulfhydrase and methionine γ-lyase are two key enzymes in the degradation of cysteine and methionine. L-Cysteine desulfhydrase catalyzes the reaction (Kredich et al., 1973; Ohkishi et al., 1981):

L-Cysteine → pyruvate + NH_3 + H_2S

L-Cysteine desulfhydrase activity has been found in various bacteria mainly belonging to *Enterobacteriaceae* (Kredich et al., 1973; Ohkishi et al., 1981).

L-Methionine γ-lyase catalyzes the reaction (Esaki & Soda, 1987):

L-Methionine → α-ketobutyrate + NH_3 + CH_3SH

High activity for this enzyme has been found mainly in *Pseudomonas* species (Tanaka et al., 1977). Among oral bacteria L-cysteine desulfhydrase and L-methionine γ-lyase have only been demonstrated in *Fusobacterium nucleatum* (Pianotti et al., 1986).

Another enzyme of interest is L-cystine lyase which catalyzes the reaction (Smith & Hall, 1987):

L-Cystine → L-cysteine persulfide+pyruvate+NH_3

L-Cysteine persulfide may then serve as a substrate in the formation of hydrogen sulfide by the same enzyme (Smith & Hall, 1987):

L-Cysteine persulfide → pyruvate+NH_3+H_2S+S^0

L-Cystine lyase has been isolated from cabbage (Smith & Hall, 1987).

Methyl mercaptan may also be formed by bacteria by a methylation of hydrogen sulfide (Drotar et al., 1987). The responsible enzyme, thiol methyltransferase also methylates methyl mercaptan with the formation of dimethyl sulfide. Thiol methyltransferase has been found in the intestinal mucosa and liver of rat (Weisiger et al., 1980). It has also been demonstrated in human liver, placenta, kidney, lungs, intestine and red blood cells (Pacifici et al., 1991; Glauser et al., 1992). No metabolic pathway is known for the formation of dimethyl disulfide.

Biochemical and Clinical Factors Influencing Oral Malodor in Periodontal Patients

Ken Yaegaki and Kazuo Sanada†*

THE AMOUNTS OF VOLATILE SULFUR COMPOUNDS (VSC) and methyl mercaptan/hydrogen sulfide ratio in mouth air from patients with periodontal involvement were 8 times greater than those of control subjects. Our studies demonstrated that, in patients with periodontal disease: 1) the concentration of disulfide, which is converted to VSC, increased in proportion to the total pocket depth; 2) 60% of the VSC was produced from the tongue surface; 3) the amount of tongue coating was 4 times greater than in control subjects; and 4) VSC production and the methyl mercaptan/hydrogen sulfide ratio of the tongue coating were increased. 2-Ketobutyrate, which is a byproduct of the metabolism of methionine to methyl mercaptan, was higher in the saliva of patients with periodontal disease. This implies that metabolism of methionine to methyl mercaptan increases in the oral cavity of patients with periodontal pockets. Since free L-methionine, rather than protein, is the main source for methyl mercaptan, we estimated the methionine supply from the gingival fluid into the oral cavity of patients with periodontal involvement. The results showed that the ratio of methionine to whole free amino acids was significantly higher than that of cysteine. Our studies suggest that not only microorganisms, but also the tongue coating and gingival fluid are factors which enhance VSC production in patients with periodontal disease. *J Periodontol 1992; 63:783–789.*

Key Words: Periodontal pockets; saliva/analysis; methyl mercaptan; hydrogen sulfide; tongue, coated; 2-ketobutyrate/analysis; methionine/analysis; gingival fluid/analysis; sulfides, volatile/analysis.

Periodontal disease frequently involves pathological oral malodor, which is caused mainly by volatile sulfur compounds (VSC), such as hydrogen sulfide, methyl mercaptan, and dimethyl sulfide.[1,2]

From ancient times,[3,4] people have known that periodontal disease causes halitosis. Hippocrates described a remedy for pathological oral malodor[3] and said, "If the gingiva become healthy again, the offensive odor vanishes." Some scientific studies[5,6–8] conducted during the past 50 years have shown that periodontal disease causes the offensive odor. Recently, Tonzetich et al.[9–15] provided evidence that pathological malodor may accelerate periodontal disease. VSC increase the permeability of the oral mucosa and collagen solubility[9,11] and decrease protein or collagen synthesis[10,15] and thus may be considered to be involved in the pathogenesis of periodontal disease. Therefore, it is important to comprehend the factors which influence pathological oral malodor in order to conduct research into the pathogenesis of periodontal disease and develop treatments for pathological oral malodor.

Microbiological studies[16,17] have demonstrated that periodontal pathogenic microorganisms contribute to increased VSC production, in particular that of methyl mercaptan, in the oral cavity. Therefore, it has been suggested that *Fusobacterium, Porphyromonas gingivalis*, and other microorganisms have important roles in the pathogenesis of halitosis.[16,17] In order to gain understanding of the pathology of halitosis associated with periodontal disease, we have conducted studies to determine clinical and biochemical factors which accelerate the production of VSC.

Association Between Halitosis and Periodontal Status

Elevated concentrations of VSC occur frequently in mouth air from patients with periodontal disease.[8,18] Tonzetich[8] demonstrated that the VSC concentration in mouth air increased with the total pocket depth, and we found, using the gas chromatography method he developed,[2] that this was higher in patients with probing depths of 4 mm or more than in subjects with probing depths of less than 4 mm (Fig. 1).[18] In particular, the methyl mercaptan concentra-

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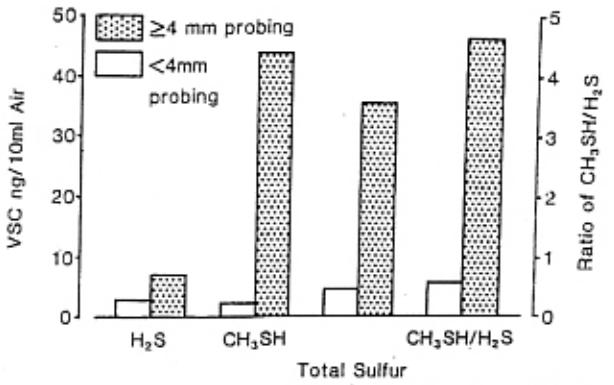

Figure 1. VSC production in mouth air from subjects (n = 17) with a gingival probing depth of 4 mm or more. In comparison with subjects (n = 14) with a probing depth of less than 4 mm, a large amount of VSC production was observed, and the methyl mercaptan/hydrogen sulfide ratio was markedly increased.

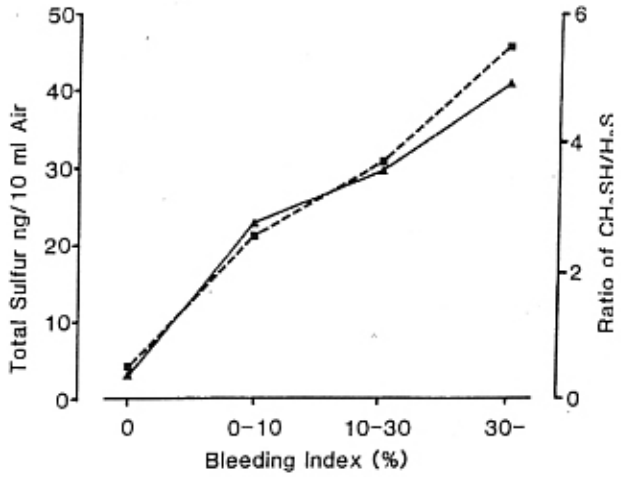

Figure 2. Total sulfur concentration and ratio of methyl mercaptan/hydrogen sulfide in relation to bleeding index. VSC production and the rat increased with bleeding index. Each value is an average of each clas the numbers in each class being as follows: 0; n = 11, 0-10; n = 7, 10 30; n = 4, 30-; n = 9.
Broken line: ratio of methyl mercaptan. Solid line: total sulfur.

tion was significantly higher in patients with periodontal disease than in controls (44.0 ng/10 ml vs. 2.6 ng/10 ml, $P < 0.01$) (Fig. 1).

The bleeding index, which is derived from the number of bleeding points caused by probings, was used to compare the extent or severity of periodontitis. Figure 2 shows that the total sulfur content in 10 ml of mouth air increased in proportion to the bleeding index.[8] The production of hydrogen sulfide in periodontal pockets was demonstrated by inserting leaded filter papers into the pockets; it must be noted that this method is not quantitative.[7] Furthermore, Coil and Tonzetich[19] have indicated increased VSC production in periodontal pockets by employing a special device to collect the gases.

It is evident that periodontal disease causes high concentrations of VSC in mouth air, with consequent quantitative changes of bad breath, although there is little information available about the qualitative change of bad breath.[18]

Since the oral environment of patients with periodontal disease may differ from healthy subjects, we wondered whether the composition of VSC associated with pathological odor was the same as that of physiological oral malodor. We determined the methyl mercaptan/hydrogen sulfide ratio by gas chromatography method.[18] These two compounds, rather than dimethyl sulfide, have been considered to be the main components of VSC in mouth air.[2,20] We found that the methyl mercaptan/hydrogen sulfide ratio in patients with probing depths of 4 mm or more was much higher than that of controls (4.64 vs. 0.58, $P < 0.01$) (Fig. 1),[18] and that it increased in proportion to the bleeding index which reflects the extent of periodontal disease (Fig. 2).[18]

The maximum probing depth was determined and compared with the methyl mercaptan/hydrogen sulfide ratio in each patient with periodontal disease. Figure 3 shows that the ratio increased with probing depth.[18] The group with probing depths of 3 mm or less had a ratio of 0.37 ± 0.10 (mean ± SE, n = 9); with 4 mm probing depths the ratio

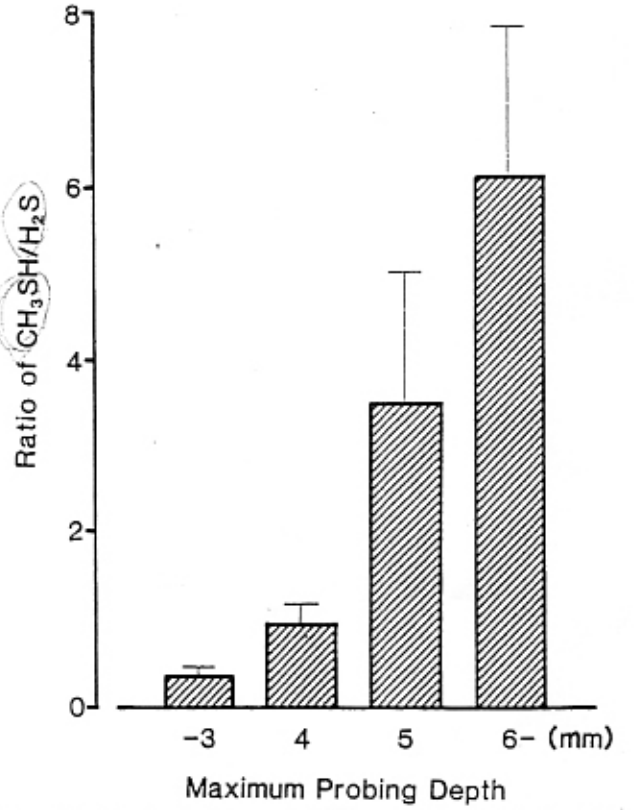

Figure 3. Ratio of methyl mercaptan/hydrogen sulfide and maximum prob ing depth in each subject. The ratio increases with depth. The number in each class are as follows, 0-3 mm, n = 9; 4 mm, n = 6; 5 mm, n = 8; 6 mm-, n = 11.

was 0.96 ± 0.23 (mean ± SE, n = 6); it was 3.49 ± 1.48 (mean ± SE, n = 8) with 5 mm depths; and 6.10 ± 1.71 (mean ± SE, n = 11) with depths in excess of 5 mm. Therefore, these results indicate that methyl mercaptar

is the main component of VSC in patients with periodontal involvement, hydrogen sulfide is the main constituent in orally healthy subjects, and the methyl mercaptan/hydrogen sulfide ratio increases with the periodontal disease severity. It can be implied from these results that estimating the amount of hydrogen sulfide, rather than methyl mercaptan, would be appropriate for evaluation of physiological malodor, especially in patients with strong psychosomatic tendencies because usually it appears to be only hydrogen sulfide that causes the objectionable smell in such patients.[21] Recently, portable bad breath detectors[‡§] have been introduced into the clinical field.[21-23] Since all of these cannot distinguish between hydrogen sulfide and methyl mercaptan, they are not applicable to our purpose. Although some portable detectors are less reliable than gas chromatography, one[1,21,23] may be useful for the screening of malodor in clinics.

We found that the methyl mercaptan concentration in mouth air from patients with periodontal disease increased compared with normal subjects. It is believed that hydrogen sulfide is produced from thiols, such as cysteine,[24,25] and methyl mercaptan originates from methionine.[17] However, it has been suggested that hydrogen sulfide can be converted to methyl mercaptan in the oral cavity.[26] We investigated whether methionine metabolism, outlined below,[17] is enhanced in the oral cavity of patients with periodontitis.

methionine→ 2-ketobutyrate + ammonia + methyl mercaptan

2-Ketobutyrate is a byproduct of the methyl mercaptan production process, so we estimated its concentration in saliva with the high performance liquid chromatography (HPLC) procedure we have developed. The saliva samples were obtained from the mandibular mucobuccal fold. The protein was removed by freezing, and then adding perchloric acid to a final concentration of 5%. The 10,000 × g supernatant was then degassed by aspiration for 5 minutes. Water was added to adjust the original sample volume, 25 µl samples were added with 50 µl 1 M acetate buffer (pH 5.0), and the sample was derivatized with 20 µl 0.1% w/v 3-methyl-2-benzothiazoline hydrazone at room temperature for 30 minutes. The HPLC conditions used were as follows: a Pico tag column[¶] was eluted at 38°C with buffer A, which consisted of 0.05% triethyl amine, and 5.0% acetonitrile buffered at pH 5.5. The column was eluted at a flow rate of 0.5 ml/min for 4 minutes; after which the Pico tag column was eluted with buffer B (60.0% acetonitrile). The acetonitrile was increased to 100% for 30 seconds, and peaks were detected and recorded at an absorbance wave length of 254 nm. The results demonstrated that 2-ketobutyrate increased dramatically in the mixed saliva from patients with periodontal disease compared with normal subjects (Table 1), and support strongly the observations that the production of methyl mercaptan from methionine is accelerated in patients with periodontal disease.

‡GC Co., Tokyo, Japan.
§Tokuyama Soda Co., Tokyo, Japan.
‖Interscan Co., Chatsworth, CA.
¶Waters Co., Milford, MA.

Table 1. 2-Ketobutyrate in Saliva

	Periodontitis (n=38)	Health (n=16)	
2-Ketobutyrate (nmole/ml)	32.7±4.1	0.6±0.5	$P<0.001$
Total pocket depth (mm)	202.7±23.6	—	

*Unpaired *t*-test.

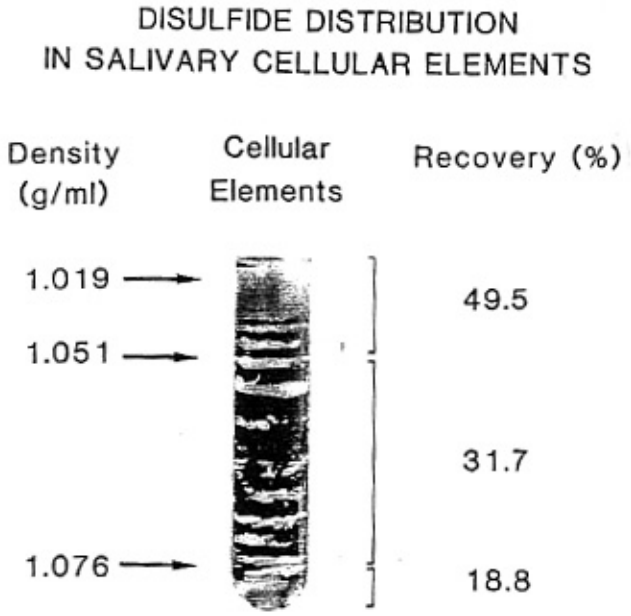

Figure 4. Distribution of disulfide in salivary cellular elements. Cellular elements were fractionated by percoll gradient centrifugation. Almost 50% of disulfide was distributed in the lower-density fraction (less than 1.051 g/ml), consisting of relatively intact cells, in comparison with other fractions.

Saliva

It has been suggested that saliva is one of the main sources of bad breath, because VSC are produced from proteins and sulfur-containing amino acids present in saliva.[1,20,24,27]

Since filtration removes 90% of thiols and disulfide from whole saliva, though dialysis does not affect the thiol and disulfide content in the saliva, and sonication of whole saliva increases these concentrations in the supernatant, Tonzetich and Johnson[24] concluded that the cellular elements of saliva are the principal sources of the thiol and disulfide groups utilized for the production of hydrogen sulfide.

We analyzed the distribution of thiols and disulfide in salivary cellular elements with a percoll density gradient centrifugation technique[28] and found that disulfide is distributed mainly in the intact cells (Fig. 4), whereas the thiol concentration was lower in intact than damaged cells. A saliva putrefaction study indicated that cell destruction increased the specific gravity of the cells and decreased disulfide content.[28,29] In light of these results, we postulated

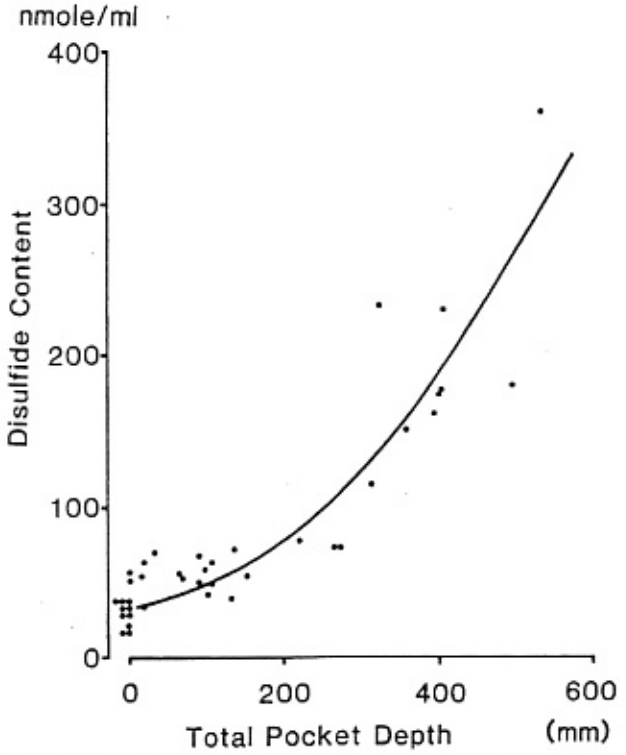

Figure 5. Disulfide in saliva and periodontal disease. Disulfide content (nmol/ml) in saliva was increased with total pocket depth in each patient. A regression curve was obtained, $Y = 40.5275 + 0.0299X + 0.0009X^2$, with a coefficient of determination of 0.8407 ($P < 0.01$).

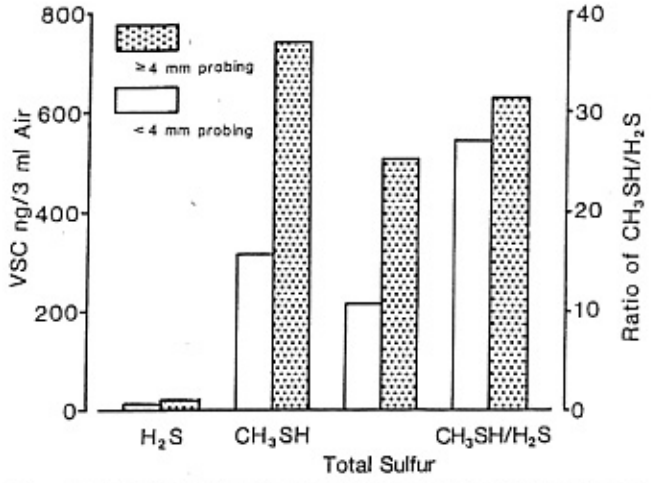

Figure 6. VSC production in a saliva putrefaction system. VSC production in saliva from subjects (n = 17) with a probing depth of 4 mm or more is almost double that of the control, but the ratio of methyl mercaptan is the same as the control average.

that the contents of the intact cells would be very rich sources of VSC.

The cellular elements in saliva comprise epithelial cells released from the oral mucosa, microorganisms, and leukocytes.[30] The epithelial cells contain keratin, cysteine-rich protein, although keratinization is not complete. Leukocytes also possess large quantities of sulfur-containing amino acids, which could be utilized for VSC production.[30] Since leukocytes migrate from periodontal pockets, their number in saliva increased in patients with periodontal disease, although epithelial cells did not increase significantly.[30] Furthermore the number of leukocytes correlated with some periodontal evaluations[30] and methyl mercaptan concentrations in mouth air. It has thus been suggested that leukocytes may increase the source of VSC in saliva of patients with periodontitis.

Our study indicated that the salivary thiol concentration is not increased significantly in patients with periodontal disease.[25] Thiol is probably too reactive to exist in its free form for long, therefore, no significant difference between the thiol concentrations of control and periodontitis groups would be found. However, disulfide is more stable than thiol, and we found that the disulfide content of saliva correlated with the severity of periodontal disease (Fig. 5).[25] A regression curve was obtained ($Y = 40.5275 + 0.0299X + 0.0009X^2$, with a correlation coefficient of 0.8404, $P < 0.01$). The disulfide content correlated also with the pocket share ratio and mean pocket depth.[25] Therefore, on the basis of these results, the salivary disulfide concentration appears to reflect the periodontal status. There is a plausible explanation for such an increase of disulfide: an increase of the salivary protein content may increase the available disulfide in saliva, as the sulfur-containing amino acids of protein molecules comprise disulfides. However, our results demonstrated that the disulfide concentration (/mg protein) also increased in proportion to the severity of periodontal disease (data not shown). Therefore, we speculated that the content of sulfur-rich proteins or sulfur-containing amino acids increased in saliva from patients with periodontal involvement.

Our study demonstrated that VSC concentrations and the methyl mercaptan/hydrogen sulfide ratio increased in mouth air in proportion to the bleeding index. As the bleeding index value and concentration of blood are mutually related, it has been suggested that other blood components, as well as leukocytes, originating from periodontal pockets may participate in acceleration of VSC production. Gibbons and MacDonald[32] found that most strains of *Porphyromonas gingivalis*, including some periodontally pathogenic strains, required hemin for their growth, and the growth rate increased in proportion to the hemin concentration. Therefore, hemin originating from blood may enhance the bacterial activity, which produces methyl mercaptan in saliva or periodontal pockets.

A saliva putrefaction system has been employed[18] to estimate whether saliva from patients with periodontitis contributes to accelerated VSC production. The head-space air in the Teflon-coated glass tube (17.5 ml volume) was replaced with nitrogen gas, 1 ml saliva was incubated for 24 hours at 37°C, and 3 ml head-space air was analyzed by gas chromatography. Figure 6 shows that the amount of VSC in head-space air from incubated saliva of patients with periodontal disease increased compared with normal subjects. However, this increase is very slight compared

with that in mouth air (Figs. 1 and 6). The methyl mercaptan/hydrogen sulfide ratio in saliva from patients with periodontitis was not different from the ratio in control, although the ratio increased dramatically in mouth air from patients with periodontal disease. There is no doubt that the elevated putrefaction activity of saliva contributes to increased VSC production in periodontal disease, but the contribution of saliva to the increased methyl mercaptan/hydrogen sulfide ratio was less than we had expected. Therefore it has been strongly speculated that some factors other than saliva may influence methyl mercaptan production in mouth air from periodontal patients.

Tongue Coating

Kaizu[33] had suggested that the tongue coating may not play an important role in the production of VSC in patients with periodontal disease, whereas removal of the tongue coating did reduce VSC in orally healthy subjects.[8,33] It has been reported that tongue coating removal does not prolong suppression of methyl mercaptan production in patients with periodontal disease.[33] However, these studies combined oral rinsing or tooth brushing with tongue coating removal, and, therefore, the amount of VSC produced by the tongue coating was not estimated accurately.

A preliminary study demonstrated that the tongue coating volume tended to increase in cases with periodontal involvement.[34] The tongue coating comprises epithelial cells released from the oral mucosa, microorganisms, and leukocytes from periodontal pockets.[34,35] As these cells, in particular leukocytes, are increased in saliva from patients with periodontal disease and accumulate on the tongue surface, tongue coating would be expected to be increased in patients with periodontal disease. Thus we have postulated that the tongue coating may be an important factor in pathological and physiological oral malodor, contrary to this previous study. Therefore, the following experiments were performed to determine the effect of tongue coating on VSC production in patients with periodontal disease.[18]

The subjects were instructed to abstain from oral hygiene, including oral rinsing and ingestion of food and liquid, on the morning of the test. After VSC analysis, cotton rolls were put around the tongue to exclude moisture and saliva; saliva on the dorsal surface of the tongue was removed with a stream of air and pure pulp tissue paper;* the tongue coating from the terminal sulcus to the apex of the tongue was removed carefully with a tongue scraper of the small spoon type to avoid contaminating the oral cavity and saliva with coating material; and the dorsal surface was cleaned with cotton pellets immersed in physiological saline. The VSC analyses of mouth air were repeated after removal of the tongue coating. Unlike previous studies of tongue coating,[8,33-35] which have usually employed classification by inspection to estimate the volume of tongue coating on the tongue surface, we measured the wet weight

*Kim Wipe, Jujoh-Kimberly Co., Tokyo, Japan.

Table 2. VSC Production from Tongue Coating

	Wet Weight (mg)	VSC (ng/10ml)	CH_3SH/H_2S Ratio
Control (n=6)	14.6	4.3 (8.3*)	1.0
Periodontitis (n=17)	90.1	18.6 (36.5*)	31.3

*Total VSC concentration in mouth air before tongue coating removal.

of the tongue coating. The VSC production by the tongue coating was calculated by subtracting the amount of VSC produced after tongue cleaning from the initial amount prior to cleaning. Table 2 shows that the group with periodontal disease (> 4 mm probing depth) had far more tongue coating than the controls (14.6 mg vs. 90.1 mg, $P < 0.01$), although the salivary flow rates of the two groups did not differ, and VSC production by the tongue coating of periodontal disease patients was estimated to be more than 4 times that of the controls (< 4 mm probing depth). Furthermore, the methyl mercaptan/hydrogen sulfide ratio was much higher in patients with periodontal disease than in controls (31.3 vs. 1.0, $P < 0.01$). It also has been demonstrated that much more VSC, especially methyl mercaptan, was produced on the tongue dorsal surface in patients with periodontal disease than in orally healthy subjects, and immediately after removal of the tongue coating the amount of VSC was reduced by almost half the initial amount. Therefore, the tongue coating must be an important factor which accelerates VSC production in patients with periodontal disease as well as in orally healthy subjects.

As the VSC composition produced by the tongue coating changed dramatically in patients with periodontal disease, these results indicate that the composition of precursors of VSC or bacterial characteristics of the tongue surface may differ in patients with periodontal disease from those in orally healthy subjects.

Gingival Fluid

Methyl mercaptan production from methionine is accelerated in mouth air from patients with periodontal disease. Tonzetich and McBride[6] demonstrated that a pathogenic strain of *Porphyromonas* produced 12 times as much methyl mercaptan as hydrogen sulfide. This study implies that periodontal pathogenic microorgansims do accelerate production of methyl mercaptan in mouth air.

Moreover, there is little information in the literature concerning the relationship between precursors of VSC and periodontal disease.[25] We found that the salivary disulfide content increased in patients with periodontal disease, but as disulfide originates from cysteine or cystine, the disulfide content would have no effect on methyl mercaptan production.

The VSC concentrations in mouth air at 2 minutes after mouth rinsing with mouthwashes, such as L-cysteine (2 mM), L-methionine (2 mM), and 0.5% casein have been

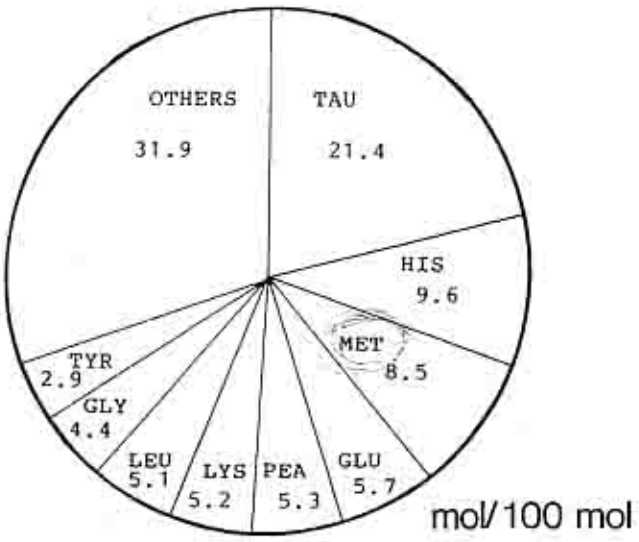

Figure 7. Amino acid composition of gingival fluids from patients with periodontal disease. Taurine (TAU) ratio is markedly high in comparison with others (n = 23).

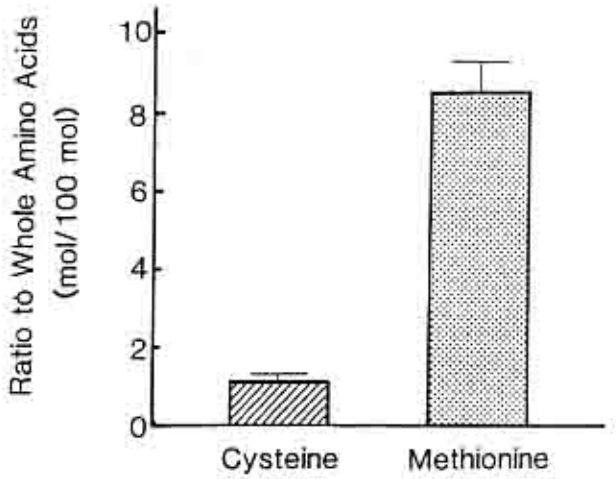

Figure 8. Methionine and cysteine ratios in gingival fluids from patient with periodontal disease. Methionine concentration is overwhelmingly higher than that of cysteine. Cystine value expressed as cysteine equivalent is also included.

determined.[36] It has been demonstrated that only free methionine is a good source of methyl mercaptan in vivo, so we investigated the free methionine supply for methyl mercaptan production. The concentration of methyl mercaptan in mouth air correlated with the severity of periodontal disease, which increased the gingival crevicular fluid flow rate. Therefore, it has been speculated that gingival crevicular fluid may be a continual supply of free methionine and other VSC precursors in patients with periodontal involvement.

The free amino acid composition of gingival crevicular fluid from the periodontal pocket was determined using a Wakosil-PTC column** with gradient system, which we developed to separate standard AN-type amino acids from physiological free amino acids. Samples were collected from the upper incisors pockets with filter strips, the papers were immersed in 100 μl ice-cold water, and sonicated. The protein was removed by Sep-pack C18[1] and samples were derivatized. We found that 99% of methionine and cysteine were recovered in this system. The results showed that taurine was present in the greatest amounts among the free amino acids in gingival fluid (Fig. 7). There are many metabolic pathways of cysteine, but two are believed to predominate. One produces pyruvic acid, ammonium ions, and hydrogen sulfide, and the other taurine. The latter pathway may be more active in the periodontal pockets or tissues.

The ratio of methionine to whole free amino acids in gingival fluid from periodontal pockets was significantly higher than those of cysteine and cystine (Fig. 8). This suggests that gingival fluid is a good source for methyl mercaptan rather than hydrogen sulfide production.

We concluded that the periodontal pocket possesses appropriate conditions for methyl mercaptan production, such as periodontal pathogenic microorganisms and an adequate supply of free methionine. Coil and Tonzetich[19] reported that very high concentrations of dimethyl sulfide, to which methyl mercaptan was dimerized, occurred in periodontal pockets. This supports the finding that methionine metabolism is accelerated in periodontal pockets by the factors mentioned above. The elevated methyl mercaptan production in periodontal pockets then would accelerate periodontal disease, since methyl mercaptan has been demonstrated to be more cytotoxic than hydrogen sulfide.[11,37]

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Conclusion

Production of VSC in patients with periodontal disease occurs in periodontal pockets and on the tongue surface. However, saliva may make only a minor contribution to pathological odor production. Methyl mercaptan, rather than hydrogen sulfide, is the main component of pathological malodor, whereas the converse applies in orally healthy subjects. The salivary disulfide concentration correlates with the severity of periodontitis, but may not contribute to the increased methyl mercaptan concentrations. The levels of 2-ketobutyrate are increased in the oral cavity of patients with periodontal disease, which indicates that the metabolism of methionine to methyl mercaptan is accelerated. The supply of methionine from gingival fluid is greater than that of cysteine or cystine.

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Desulfuration of Cysteine and Methionine by *Fusobacterium nucleatum*

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Fusobacterium nucleatum *is a Gram-negative anaerobic rod-shaped bacterium frequently isolated from human dental plaque. It is capable of the desulfuration of cysteine and methionine, resulting in the formation of sulfide and thiol volatiles, respectively. Intact cells, as well as cell-free extracts produced by French pressure cell lysis of* F. nucleatum, *hydrolyzed radiolabeled cysteine to produce sulfide, pyruvic acid, and ammonia. The hydrolysis products of radiolabeled methionine were a volatile thiol, ketobutyrate, and ammonia. Both activities were associated with the cytoplasmic component, not the membrane. The desulfuration mechanisms are heat-labile, inhibited by the presence of excess substrate, and rates are dependent upon substrate concentration. These dissimilar pathways by* F. nucleatum *can account in part for the presence of sulfur-containing volatile products that occur in the mouth.*

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Introduction.

The genus *Fusobacterium* includes several species of obligately anaerobic, Gram-negative rods indigenous to the oral cavity in man and animals. The number of fusobacteria per milliliter of saliva has been estimated to be 5.6×10^4, and they account for about 3 to 4% of the total cultivable anaerobic isolates from dental plaque and gingival crevice areas of man (Gibbons *et al.*, 1963; Slots, 1979). Moore *et al.* (1982a) indicated that this species represented 4.4% of the total gingival isolates and 4.8% of total genus *Fusobacterium* from young adult humans with experimental gingivitis. Moore *et al.* (1982b) further indicated that *F. nucleatum* was present in 85% of samples taken from 34 affected subgingival sites with severe periodontitis. In addition, Berglund (1971) showed that patients with periodontal disease demonstrate significantly elevated serum antibody titers to *F. nucleatum*. This bacterium exhibits biological activities related to the etiology of gingival inflammation, oral disease, and fetid breath, insofar as it attaches to and lyses human erythrocytes (Falkler *et al.*, 1983), produces large amounts of collagenase (Sveen and Skaug, 1980), butyric acid, and volatile sulfur compounds (Kostelc *et al.*, 1980). Volatile sulfur, the principal malodorous component present in fetid mouth air, is derived from the degradation of sulfur-containing substrates (leucocytes, epithelial cells, food debris) in whole saliva and saliva sediment. These cellular elements of saliva are the principal substrates used by bacteria for the production of hydrogen sulfide and methyl mercaptan (McNamara *et al.*, 1972; Tonzetich, 1977; Solis-Gaffar *et al.*, 1979; Shomers *et al.*, 1982).

In this study of *F. nucleatum*, we investigated metabolic pathways that result in the formation of volatile sulfur compounds. Specifically, immediate end-products of L-cysteine and L-methionine dissimilation were identified and quantitated, and the cellular localization of these enzymatic activities was determined.

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Materials and methods.

Preparation of cell suspensions. — *Fusobacterium nucleatum ATCC25586* was routinely stocked in chopped meat broth and grown in 300-mL cultures of the Peptone Yeast Extract Basal Medium described by Holdeman *et al.* (1977). L-methionine or L-cysteine-HCl monohydrate, 10 mmol/L, was included in the basal medium as indicated in the experiments. Media preparation, inoculation, cell growth, and cell harvest were conducted under anaerobic gas. Cells were harvested in stoppered tubes by centrifugation following 96 hours of growth at 37° C (A_{620} *ca.* 1.10), then thoroughly washed three times with 0.05 mol/L Tris-HCl buffer (pH 7.4) containing 0.01 mol/L $MgCl_2$ and 1 mmol/L dithiothreitol, and re-suspended in the buffer to 5% of the original volume of culture. The cell suspension was stored frozen in aliquots under nitrogen at −70° C until use.

Preparation of cell fractions. — Cells were suspended in buffer containing 0.5 mg/mL bovine serum albumin and passed through a French Pressure Cell (American Instrument Co., Silver Spring, MD) at 24,000 pounds per square inch until greater than 95% cell breakage had occurred as determined by phase contrast microscopy. Whole cells and debris were removed by centrifugation at 2800 *g* for 10 min at 5° C, and a portion of the supernatant fluid (cell-free extract) was stored in aliquots at −70° C. The remaining supernatant was further centrifuged at 100,000 *g* for two hours to recover clear cytoplasmic supernatant and a pellet containing membranes. The membranes were washed once in a small volume of Tris buffer and then recovered by centrifugation. Each of the cell fractions—*i.e.*, cell-free extract, cytoplasm, and membrane—was stored under nitrogen at −70° C.

Analytical procedures. — Cell-free extracts were incubated anaerobically for 40 min at 37° C in stoppered 85 × 11 mm test tubes containing 0.1 mol/L Tris buffer (pH 8.4) and 4 mmol/L cysteine·HCl or L-methionine and water in a final volume of 2.0 mL. Soluble sulfide produced was assayed by means of the dimethyl-p-phenylenediamine, $FeCl_3$ colorimetric method as described by Siegel (1965). Total pyruvate formation was measured through the formation of the dinitrophenylhydrazone derivative as described by Kredich *et al.* (1972). Alpha-ketobutyrate was measured with the colorimetric method of Katsuki *et al.* (1971). Total pyruvate produced by dissimilation of 10 mmol/L L-(U-^{14}C) cysteine·HCl (0.25 mCi/mmol), as well as the total alpha-ketobutyrate from 10 mmol/L L-(U-^{14}C) methionine (0.25 mCi/mmol) (Amersham, Arlington Heights, IL), was quantitated by reaction with 2,4,-dinitrophenylhydrazine to form the radiolabeled hydrazone, which in turn was extracted into ethyl acetate and counted in a scintillation spectrometer.

In order to establish the qualitative differences between ketoacid products, we spotted the hydrazones onto thin layer chromatography plates coated with Silica Gel B. The plates were developed in an ascending manner with chloroform-carbon tetrachloride (2:1 v/v), then dried at room temperature and re-developed in the same dimension with chloroform-dioxane (94:6 v/v). The phenylhydrazone spots were developed by

spraying the plates with 95% ethanol:7 mol/L KOH in H_2O (1:1 v/v), and comparisons of R_f were made against reference compounds.

The concentration of ammonia was determined by the glutamic dehydrogenase method described by Su *et al.* (1969). Appropriate controls and standards were included in all analytical assays.

Radiolabeled volatile sulfides were produced by reaction mixes incubated in Warburg flasks (Huisingh and Matrone, 1972) equipped with center well and double side-arm venting plugs. The main compartment contained up to 0.50 mL cell extract, 0.10 mol/L Tris-HCl buffer (pH 7.4), 1 mmol/L dithiothreitol, and 20 mmol/L (^{35}S) cysteine or (^{35}S) methionine (Amersham, Arlington Heights, IL) at 0.25 mCi/mmol. The final reaction volume of 2.0 mL was obtained by addition of distilled water. Phosphoric acid (0.2 mL of 10 mol/L) used to stop the reaction was stored in a side bulb. Whatman #1 filter paper (25 × 50 mm), accordian-folded and soaked with 0.2 mL of 25% w/v zinc acetate, was placed in the center well for quantitative precipitation of the sulfide-containing gases generated during incubation. The flasks were flushed with nitrogen and capped with gas-tight stoppers. After 30 minutes' incubation, the reaction was stopped, and any sulfide remaining in solution was liberated by the tipping of the phosphoric acid into the main compartment. The flasks were further incubated for 10 min to ensure trapping of all volatile sulfides. The filter papers were removed and placed into 15 mL of aqueous counting scintillant for counting in a scintillation spectrometer.

Other methods. — Cell protein levels were measured by Lowry *et al.* (1951) determination on trichloroacetic acid precipitates. The apparent K_m and V_{max} were determined in triplicate determinations from Lineweaver-Burk plots.

Results.

Evidence that sulfur-containing amino acids are directly degraded by *F. nucleatum* cells to form volatile sulfides is shown in Table 1. The data indicate that a minuscule amount of carbon-containing sulfide end-product was precipitated onto a zinc acetate trap when ^{14}C L-cysteine was present as the sulfur-bearing substrate. However, with ^{14}C L-methionine as substrate, there was the precipitation of ^{14}C-containing end-product. The data in Table 1 further indicate that both ^{35}S-L-cysteine and ^{35}S-L-methionine are desulfurated to form volatile sulfide end-products. This preliminary evidence is interpreted to indicate that L-cysteine degradation led to the formation of non-carbon-containing volatile sulfur compound, hydrogen sulfide. Conversely, L-methionine degradation led to the formation of a carbon-containing end-product, methyl mercaptan.

Identification of the end-products of L-cysteine HCl and L-methionine desulfuration is presented in Table 2. Cell-free extracts of *F. nucleatum* were incubated in stoppered test tubes containing buffered solutions of either one of the sulfur amino acids. Sulfide, pyruvic acid, and ammonia were found as products of cysteine desulfuration. Ketobutyric acid and ammonia were found as products of methionine desulfuration. That ketobutyric acid derived from methionine, and the pyruvic acid from cysteine, were further confirmed by thin layer chromatography against known standards (data not shown). Previous studies from our laboratory (Mink *et al.*, 1983) showed the volatile sulfur compound production capacity of *F. nucleatum* and other oral anaerobic bacterial species. After good growth was obtained in the Peptone Yeast Extract Medium, culture head space was analyzed for sulfide content by gas chromatography. The results demonstrated that members of the genus Fusobacterium produced several-log-higher hydrogen sulfide (H_2S) and methylmercaptan (CH_3SH) levels than other non-treponemal bacterial species analyzed.

The association of volatile sulfide production with enzyme activity is indicated by the results of experiments presented in the Fig. Production of end-product (sulfide) by a constant amount of extract prepared by French pressure cell lysis was studied as a function of substrate concentration. Radiolabeled sulfur (^{35}S) accumulation was monitored in the zinc acetate trap. The release of ^{35}S from cysteine or methionine was dependent upon substrate concentration. Further indication that the desulfuration mechanism is enzyme-mediated was the loss of activity upon the heating of reaction mixtures at 100° C for 10 min (Table 1).

The relative affinities (K_m values) were determined in replicate experiments by measuring the rate of ketoacid formation as a function of different concentrations of L-cysteine or L-methionine in mixtures with cell-free extracts. The mean (n = 3) K_m for L-cysteine calculated from the Lineweaver-Burk plot was 3.16 mmol/L (V_{max}, 1.24 nmol/min) and for L-methionine 5.57 mmol/L (V_{max}, 0.42 nmol/min). It is evident that there exists a moderate affinity of enzyme for its substrate.

A comparison of the rate of ketoacid formation by each of the principal cell fractions, as well as growth medium, shown in Table 3, indicates that the greatest activity was present in the cytoplasm fraction, not the membrane portion of the fractionated cells. Extracellular enzymatic activity was not detectable in the spent growth medium.

The inhibition of enzyme by excess substrate was used to establish presumptive evidence for a cysteine desulfhydrase separate from methionine desulfhydrase (methionase). Radiolabeled U-^{14}C-L-cysteine (10 mmol/L) was incubated with cytoplasm containing excess (100 mmol/L) L-methionine; radiolabeled U^{14}C-L-methionine (10 mmol/L) was incubated in cytoplasm containing excess (100 mmol/L) L-cysteine. In this way, if only one enzyme existed, the reaction of ^{14}C amino

TABLE 1
PRODUCTION OF SULFIDES FROM RADIOLABELED SUBSTRATES BY *F. nucleatum*

	Counts Per Minute			
Substrate	Cell Suspension (37° C, 10')	% of Total Counts	Cell Suspension (100° C, 10')	% of Total Counts
L-cysteine (U^{14}C)	131	0.01	53	0.004
L-cysteine (^{35}S)	55,925	8.2	3,692	0.50
L-methionine (U^{14}C)	7,183	3.2	90	0.03
L-methionine (^{35}S)	61,743	8.5	1,269	0.17

Reaction mixture devoid of cellular material: L-cysteine (U^{14}C) 167 cpm, L-cysteine (^{35}S) 350 cpm, L-methionine (U^{14}C) 105 cpm, and L-methionine (^{35}S) 539 cpm.

TABLE 2
HYDROLYSIS OF L-CYSTEINE HCl AND L-METHIONINE BY CELL EXTRACT OF *F. nucleatum*

			Specific Activity*		
Culture Medium	Substrate	Sulfide	Pyruvate	Keto-butyrate	NH_3
Base Medium with 10 mmol/L Cysteine HCl	L-cysteine HCl	49.5	8.2	ND**	16.0
Base Medium with 10 mmol/L Methionine	L-methionine	ND	ND	28.0	16.0

*Nanomoles min^{-1} mg^{-1} cell extract protein.
**None Detected.

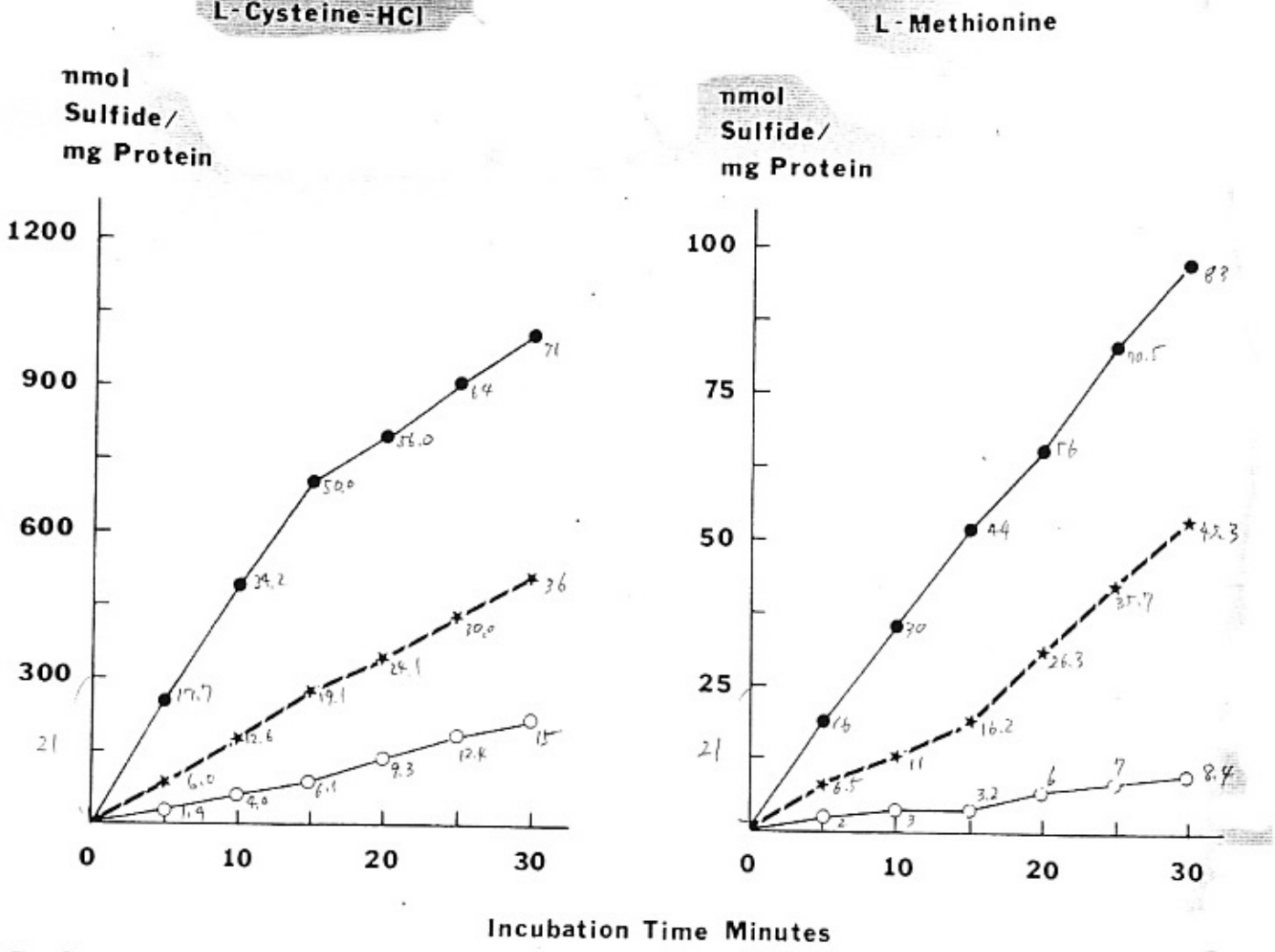

Fig.—Production of H_2S (from cysteine) and CH_3SH (from methionine) *vs.* time by buffered *F. nucleatum* cell extract. Closed circles represent 0.5 mmol/L, star represents 2.0 mmol/L, and open circle represents 6.0 mmol/L substrate.

TABLE 3
CELLULAR LOCATION OF CYSTEINE AND METHIONINE DISSIMILATORY ACTIVITY

Fraction Tested	Specific Activity* L-Cysteine	L-Methionine
Spent Broth	ND**	ND**
Cell-free Extract	8.2	16.8
Cytoplasm	17.2	30.7
Membrane	ND	ND

*Nanomoles Keto acid min^{-1} mg^{-1} protein.
**No end-product detected.

acid degradation would be precluded by the saturating concentration of competing unlabeled substrate. The specific activity {nmoles ketoacid·min^{-1}·(mg cytoplasm protein)$^{-1}$} for ^{14}C L-cysteine hydrolysis was 34.0, and in the presence of excess methionine the cytoplasmic activity was 23.5. The specific activity for hydrolysis of ^{14}C L-methionine was 27.4 units, and 15.4 units in the presence of excess L-cysteine. Therefore, a saturating concentration of competing substrate did not prevent desulfuration of demethiolation of L-cysteine or L-methionine. This observation, coupled with the observed differences in the keto acid formed from cysteine or methionine, and the different K_m values, indicates the presence of separate enzymes.

Beta-cystathionase catalyzes conversion of L-cystathionine to homocysteine, pyruvate, and ammonia. The enzyme does not act directly on cysteine or other thiols; nevertheless, it may account for cysteine desulfhydrase activity in bacteria. Incubation mixtures containing washed cysteine- or methionine-grown cells, and the substrate L-cystathionine (2 mmol/L), Tris-HCl buffer (10 mmol/L), pH 8.4, and dithiothreitol (1 mmol/L) did not result in the formation of pyruvate or ammonia. Failure to hydrolyze the substrate is indicative of the absence of cystathionase.

Discussion.

Through a combination of radiochemical methods with conventional chromatographic and spectrophotometric procedures, the immediate end-products of L-cysteine and L-methionine dissimilation by *F. nucleatum* were identified and quantitated. These end-products are indicative of their route of formation.

The desulfuration of cysteine to hydrogen sulfide by extracts of bacteria is most often attributed to the action of the enzyme cysteine desulfhydrase, although more complicated pathways

may also contribute to the process. The stoichiometric equation for the cysteine desulfhydrase reaction is as follows:

$$\text{L-cysteine} \xrightarrow[\text{desulfhydrase}]{\text{cysteine}} \text{pyruvate} + \text{ammonia} + H_2S$$

This equation predicts equimolar yields of pyruvate, ammonia, and H_2S; however, in the present study a 1:1 stoichiometry in cysteine degradation products could not be demonstrated. Instead, an excess of H_2S was always found. This situation is similar to that found by Anderson and Johanson (1963) for the cysteine desulfhydrase of *E. coli* and by Guarneros and Ortega (1970) and Kredich *et al.* (1973) with *Salmonella typhimurium*.

Loesche and Gibbons (1968) reported that resting cell suspensions of *Fusobacterium nucleatum* are able to catabolize methionine with release of ammonia. Based on results of additional experiments on deamination and demethiolation of methionine by bacteria and fungi, Ruiz-Herrera and Starket (1969) showed that methionine is first deaminated and then demethiolated with the production of methanethiol and alpha-ketobutyric acid; no sulfide or hydrosulfide products were reported. The hydrolysis of methionine by *F. nucleatum*, as described in this study, resulted in the formation of a sulfidic product and keto acid. Hydrolysis of ^{35}S L-methionine by *F. nucleatum* cell suspensions and cell extracts resulted in the liberation of a ^{35}S-containing product trapped by the zinc-acetate-impregnated filter paper trap. However, qualitative analysis of incubated methionine-cell extract mixtures did not indicate that the sulfur was present as sulfide. This observation — coupled with further experimental evidence that desulfuration of ^{14}C(U)-methionine by cell fractions resulted in the formation of a radiolabeled ^{14}C-sulfur compound that complexed with the zinc-acetate paper trap — strongly indicates that methionine desulfuration released an organic thiol (mercaptan). Colorimetric and chromatographic analysis for keto acids in end-products of methionine dissimilation by cell fractions of *F. nucleatum* indicates the presence of a ketobutyric acid. Based on the overall results of experiments on demethiolation of methionine, it is concluded that L-methionine is decomposed by *F. nucleatum* as follows:

$$\text{methionine} \rightarrow \text{ketobutyric acid} + \text{ammonia} + \text{methyl mercaptan}$$

The failure of *F. nucleatum* to convert L-cystathionine to pyruvate or ammonia precludes the involvement of cystathionase in the dissimilation of cysteine or methionine.

The production of additional types of volatile sulfur, including dimethylsulfide $(CH_3S)_2$, has not been obviated by these studies. In the gas chromatographic analysis of *F. nucleatum* culture head space, dimethylsulfide concentrations, if present, were too low to permit positive identification. Further, dimethylsulfide would not react with zinc acetate, and its presence went undetected in the present study.

French pressure cell disruption and centrifugation of the cell fragments localized both cysteine and methionine dissimilatory activities in the cytoplasm. The enzymes were associated with the cytoplasm, not with the membranes or extracellular materials released into the growth medium. The activity was heat-labile and mediated by separate enzymes, as indicated by the end-product data and K_m values.

Results of the present study show that *F. nucleatum* is capable of desulfuration of cysteine and methionine, resulting in the formation of sulfide and thiol volatiles. These desulfuration mechanisms may account in part for the presence of sulfur containing volatile products that occur in the mouth.

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Relationship between volatile sulphide compounds concentration and oral bacteria species detection in the elderly

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Aim: To evaluate the relationship between oral bacteria species detection and volatile sulphide compounds (VSC) concentration in the elderly. **Design:** Cross-sectional microbiological and clinical VSC examination of elderly. **Participants:** Sixty-seven elderly people aged 75, who were functionally independent and dentate. **Methods:** VSC (H_2S and CH_3SH) concentrations in the mouth air of subjects were measured using portable gas chromatography. Oral bacteria samples were taken from dental plaque and identification of bacteria species was accomplished using standard methods. **Results:** Fewer than 20% of subjects showed more than 10ng/10ml of H_2S (severe odour level). The detection rate of *P. melaninogenica* was significantly higher in elderly people with more than 10ng/10ml (p=0.043) levels. *Fusobacterium* had a tendency to be found in those with more than 10ng/10ml, but a significant relation was not found. **Conclusions:** The results suggest that *Fusobacterium* and *P. melaninogenica* may be involved in the production of H_2S in the oral cavity of elderly people.

Key words: Volatile sulphide compounds, oral malodour, oral bacteria

In recent years concerns about oral malodour have been increasing. Oral malodour includes unpleasant smells resulting generally from periodontal disease[1–5], and more rarely from diabetic patients[6] and those with heavy caries[7]. Oral care for elderly persons is important for prevention not only of oral disease but for general disease, for example, aspiration pneumonia[8–10]. Oral malodour is unpleasant for those caring for the elderly and can therefore be a problem in performing oral care smoothly.

Volatile sulphide compounds (VSC) have been considered to be correlated with oral malodour and several VSCs have been used as the indicator of oral malodour[4,11–14]. There have been several reports on the VSC production ability of bacteria species[14–20]. But there have been no reports on the relation between oral bacteria species detection and oral VSC concentration in mouth air, and so an epidemiological study of the elderly to elucidate this was thought to be advantageous.

Dental plaque is known to be a biofilm, a reservoir of bacteria species from which plantonic bacteria disperse and attach to other organs and tissues[21]. We suggested that dental plaque, saliva, the

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tonsils and pharynx harboured similar biofilm bacteria and that these organisms were released from tooth surfaces into saliva and then colonised on the oral cavity surface[22,23]. We hypothesised that bacterial species producing VSC in periodontal pockets and the dorsum of the tongue originate in dental biofilm. Our major concern was the possible association of bacterial species in dental biofilm with VSCs concentrations in the oral cavity.

In the present study, we analysed the relationship between VSCs concentrations and bacterial species detected in the dental plaque of the elderly.

Methods

Subjects

Sixty-seven elderly people aged 75 years (44 males and 23 females) from Niigata prefecture in Japan participated in this study, which was conducted in June, 2002. All of the participants were functionally independent and dentate.

VSC analysis of mouth air

H_2S and CH_3SH concentration were used as the indicator of oral malodour. The VSC concentrations in the mouth air of subjects were measured as follows.

- Prior to each analysis, following a deep breath, the subjects were instructed to keep their mouth closed and breathe through their nose quietly for 30 sec.
- A 1ml-disposable syringe was then inserted into the centre of the oral cavity through the lips and teeth, while the mouth remained closed.
- Thereafter, after aspirating 1ml of oral air with the disposable syringe, 0.5ml of oral air was injected into the GC-SCS for portable gas chromatography (Takasago Electoric Co., Ltd, Japan, 24).
- To avoid inter-operator variation, all gas chromatography measurements were performed by a single trained individual.

Identification of oral bacteria

Samples were taken from dental plaque on upper molar teeth or upper molar portions of dentures using sterile cotton sticks. The plaque samples were placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate buffered saline) and taken to Bio Medical Laboratory (Tokyo, Japan) for analysis. For aerobic bacteria species detection and identification, each sample was poured directly onto chocolate agar, OPA staphylococcus, and drigalski agar plates (Nippon Decton Dickinson Co., Ltd, Tokyo, Japan) using a stick. The plates were incubated in an atmosphere of 5% CO_2 in H_2 at 37^0C for 24-48 hours. Representative microbial colonies from each plate were gram stained and isolated by identification of their characteristic appearance, as well as haemolytic, catalytic, and oxidase reactions[9]. Those species found in a majority of the subjects were suspended in 1ml of 0.5% saline, gently shaken, and the results are shown. The following methods were used to confirm identification of the bacteria on the detection plates: *Staphylococcus aureus* [Methicillin sensitive (MSSA) and resistant (MRSA)] by using PS latex, rabbit plasma, and MRSA screening plates (Nippon Becton Dickinson Co); *Pseudomonas sp.* by VITEK [BioMerieux Vitek Japan (BVJ), Tokyo]; *Haemophilus influenzae* (*H. influenzae*) by a Haemophilus ID4 plate (Nippon Becton Dickinson Co) and *Candida* species by Candida check (Intron Laboratories Inc., Tokyo). For anaerobic bacteria species detection and identification, each sample was poured directly onto an HK agar plate and incubated for 48–72 hour under anaerobic condition using the gas pack system. Representative microbial colonies from each plate were gram stained and isolated by RapID ANAII system. Each colony was suspended in 0.6% KCl, 0.05% $CaCl_2$, 0.16mM NaOH. The suspension was inoculated in 10 separate detection medium (1: 0.4% Urea, 2: 0.1% p-Nitrophenyl-β, D-disacharide, 3: 0.1% p-Nitrophenyl-α, L-arabinoside, 4: 0.1% p-Nitrophenyl-β, D-galactoside, 5: 0.1% p-Nitrophenyl-α, D-glucoside, 6: 0.08% p-Nitrophenyl-β, D-glucoside, 7: 0.08% p-Nitrophenyl-α, D-galactoside, 8: 0.08% p-Nitrophenyl-α, L-fucoside, 9: 0.1% p-Nitrophenyl-n-acetyl-β, D-glucosaminide, 10: 0.1% p-Nitrophenyl-phosphate) and incubated in an atmosphere of 5% CO_2 in H_2 at 37° for 4–6 hours (primary test). In a secondary test, 0.01% 3-Phenyl-methylaminoacrolein, 0.1% Hydrochloride acid, 1.0% Acetic acid was added to reaction mixture 3–9 and INNOVA Indole to reaction mixture[10]. Bacterial species were identified by both the results of primary and secondary testing. The levels of detection for each organism were determined according to the manufacturers' instructions.

Statistical methods

In the series of oral malodour measurements, several examiners detected severe odour at a concentration of about 10ng/10ml of H_2S and about 0.5ng/10ml of CH_3SH. Therefore, concentration of H_2S and CH_3SH were classified into two groups (≦10ng/10ml, 10ng/10ml< for H_2S and ≦0.5ng/10ml, 0.5ng/10ml < for CH_3SH). The relation between VSC concentration and detection of bacteria species was analysed using a gender-adjusted Mantel-Haenszel test. Difference at the .05 level was considered statistically significant. SPSS for Windows (version 10.0) was used in performing all statistical analyses.

Results

The distribution of VSC concentration

The distribution of H_2S and CH_3SH concentration is shown in *Table 1*.

Table 1 Distribution of subjects in VSC concentration by sex

(1) H_2S	Men	Women	Total
≦10ng/10ml	38 (86.4)	17 (73.9)	55 (82.1)
10 ng/10ml<	6 (13.6)	6 (26.1)	12 (17.9)
(2) CH_3SH	Men	Women	Total
≦0.5 ng/10ml	28 (63.6)	7 (30.4)	35 (52.2)
0.5ng/10ml<	16 (36.4)	16 (69.6)	32 (47.7)

Table 2 Detection rate of major microorganisms

(1) Aerobic microorganisms	Number	Percentage
α-Streptococcus	67	100
Neisseria sp.	67	100
Candida sp.	29	43.3
Corynebacterium	5	7.5
E. clocae	4	6.0
(2) Anaerobic bacteria species	Number	Percentage
Capnocytophaga sp.	67	100
P. melaninogenica	36	53.7
Fusobacterium	18	26.9
P. corporis	15	22.4
P. intermedia	6	9.0

Table 3 The relation between H_2S concentration and oral bacteria detection

	H_2S concentration ≦10 ng/10ml No. (%)	10 ng/10ml< No. (%)	OR	95% CI	*p*
Aerobic					
Candida sp.	25 (45.5)	4 (33.3)	0.562	0.147–2.118	0.394
Corynebacterium	5 (9.1)	0 (0)	*	*	
Anaerobic					
P. melaninogenica	26 (47.3)	10 (83.3)	5.305	1.052–26.759	0.043
Fusobacterium	12 (21.8)	6 (50.0)	3.506	0.939–13.090	0.062
P. corporis	12 (21.8)	3 (25.0)	1.310	0.298–5.754	0.720
P. intermecdicus	5 (9.1)	1 (8.3)	1.080	0.114–10.242	0.946

*OR and 95% CI cannot be calculated in *Corynebacterium* because this bacteria species was not detected in subjects with more than five.

Less than 20% of subjects showed severe odour levels in H_2S concentration (more than 10ng/10ml of H_2S). A greater percentage of women than men had 10ng/10ml, but no significant difference was found between genders. With regard to CH_3SH, about half the subjects had a recognition level of CH_3SH concentration (more than 0.5ng/10ml of CH_3SH). The concentration of CH_3SH of women was significantly higher than that for men.

The detection of bacterial species

The detection rate of major micro-organisms from dental plaque, as determined using the manufactures' instructions, is shown in *Table 2*. Of the aerobic micro-organisms, α-*Streptococcus* and *Neisseria* sp. were detected in all subjects. *Candida* sp. was isolated from 43.3% of subjects. The detection rate of other bacterial species was less than 10%.

Regarding anaerobic bacteria species, only *Capnocytophaga* sp. were detected in all subjects. *P. melaninogenica* was isolated from about half of subjects (53.7%). *Fusobacterium* species and *P. corporis* were isolated from about quarter of subjects. There was not significant differentiation in the detection rates of bacteria species between both sexes.

The relation between VSC concentration and detection of bacteria species

We analysed the relationship between VSC concentration and detection of each bacterial species by using Mantel-Haenszel test. Subjects with more than 10ng/10ml of H_2S showed a significantly higher detection rate of *P. melaninogenica* than subjects with less than 10ng/10ml (*Table 3*). For *Fusobacterium*, though not statistically significant, the odds of detection amongst those with more than 10ng/10ml were 3.5 times greater than the odds among the less than 10ng/10ml group (OR=3.5 and 95% CI=0.939, 13.090). Regarding aerobic micro-organism species, no significant relationships were seen. CH_3SH concentration was not related to micro-organism detection.

Discussion

Studies on the investigation of bacterial species concerned with oral malodour have mainly been performed by measuring VSC production ability of laboratory strains of oral bacteria species[15,16,18]. Recently, Awano *et al.*[25] reported the relationship between the presence of periodontopathogenic bacteria in saliva and halitosis in adults (average age: 50.0±13.5 years). Kazor *et al.*[26] found that *Atopobium parvulm*, a phylotype (clone B5095) of *Dialister*, *Eubacterium sulci*, a phylotype (clone DR034) of the uncultivated *Phylum* TM7, *Solobacterium moorei*, phylotype (clone BW009) of *Streptoccocus* were associated with halitosis in adults.

However, oral bacterial flora depends on age and oral condition. Furthermore, oral malodour of elderly people has been noticed by carers. From this point of view, the present study provides useful data to identify bacterial species relating oral malodour in the elderly.

The results of the study indicated that *P. melaninogenica* was significantly correlated with H_2S concentration and *Fusobacterium* had a tendency to be correlated with H_2S concentration. It has been reported that *Fusobacterium* has a higher H_2S production ability than other bacteria species[16,18]. These reports support our results. *Fusobacterium* are also isolated from periodontal pockets and the tongue[27–29] and are thought to be distributed in various parts of the oral cavity by means of saliva, causing oral malodour. It has been discussed that *Fusobacterium* produce H_2S in periodontal pockets and in tongue coating, as well as in dental plaque. With regard to *Prevotella* species, Paryavi-Gholami[30] reported that *P. oralis* are related to VSC levels in children. *P. oralis* was not isolated from the subjects. Oral bacteria flora changes with age, which may explain differences in bacteria species relating to oral malodour among subjects of various ages. *Fusobacterium* and *P. melaninogenica* are known to contribute greatly in forming biofilm[31]. There is a possibility that biofilms incorporate various bacterial species including some producing H_2S. Furthermore, pathogenic bacterial species are found in biofilm[22,32,33]. Oral care procedures for removing these bacterial species must be useful for preventing oral malodour as well as general disease.

No bacterial species showed significant association with CH_3SH concentration. The result may be concerned with less CH_3SH production of bacteria species than we examined, or with lower sensitivity of the detection system to the CH_3SH products.

In the present study, we have suggested which bacteria species are related to oral malodour in the elderly, as a useful indicator to help decrease oral malodour. The prevention of odour requires the removal of the biofilm from the tooth surface as well as dorsum of the tongue. For further investigation of the relationship between these bacterial species and oral malodour, establishment of a system for quantitative analysis of these bacterial species is required.

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