Effects of pH and Lactate on Hydrogen Sulfide Production by Oral Veillonella spp.

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Indigenous oral bacteria in the tongue coating such as Veillonella have been identified as the main producers of hydrogen sulfide (H$_2$S), one of the major components of oral malodor. However, there is little information on the physiological properties of H$_2$S production by oral Veillonella such as metabolic activity and oral environmental factors which may affect H$_2$S production. Thus, in the present study, the H$_2$S-producing activity of growing cells, resting cells, and cell extracts of oral Veillonella species and the effects of oral environmental factors, including pH and lactate, were investigated. Type strains of Veillonella atypica, Veillonella dispar, and Veillonella parvula were used. These Veillonella species produced H$_2$S during growth in the presence of L-cysteine. Resting cells of these bacteria produced H$_2$S from L-cysteine, and the cell extracts showed enzymatic activity to convert L-cysteine to H$_2$S. H$_2$S production by resting cells was higher at pH 6 to 7 and lower at pH 5. The presence of lactate markedly increased H$_2$S production by resting cells (4.5- to 23.7-fold), while lactate had no effect on enzymatic activity in cell extracts. In addition to H$_2$S, ammonia was produced in cell extracts of all the strains, indicating that H$_2$S was produced by the catalysis of cystathionine γ-lyase (EC 4.4.1.1). Serine was also produced in cell extracts of V. atypica and V. parvula, suggesting the involvement of cystathionine β-synthase lyase (EC 4.2.1.22) in these strains. This study indicates that Veillonella produce H$_2$S from L-cysteine and that their H$_2$S production can be regulated by oral environmental factors, namely, pH and lactate.

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ral malodor is due to metabolic products of bacteria in the oral cavity, particularly those living on the dorsum of the tongue (1, 2). Some cases of oral malodor are known to be linked with periodontitis (3, 4), and thus various periodontitis-related bacterial species have been detected in the tongue coating (5, 6). These findings also suggest that the tongue coating plays a role in the reservoir of such bacteria (5). Most of these bacteria have the ability to produce hydrogen sulfide (H$_2$S), one of the major components of oral malodor (7, 8). In a previous study (9), we focused on oral malodor in patients without oral diseases such as periodontitis or caries and found that the predominant H$_2$S-producing bacteria were not periodontitis-related bacteria but were mainly indigenous bacteria of the oral cavity such as Veillonella and Actinomyces. Among these, Veillonella species, including V. atypica, V. dispar, and V. parvula, were dominant (9). Veillonella species are Gram-negative anaerobic micrococci that are frequently detected in the tongue coating (6, 9). These bacteria are asaccharolytic but utilize lactate, pyruvate, and oxaloacetate as energy sources. Although several studies have reported that Veillonella species produce H$_2$S (1, 8, 10, 11), the metabolic properties of H$_2$S production have not been fully understood. In the tongue coating, environmental factors such as lactate concentration and pH change continuously, suggesting that such environmental changes may affect the activity of H$_2$S production by Veillonella.

In the present study, therefore, we investigated the metabolic properties of the H$_2$S production of oral Veillonella species by the use of growing cells, resting cells, and cell extracts of Veillonella atypica, Veillonella dispar, and Veillonella parvula and the effects of oral environmental factors, namely, pH and lactate, on H$_2$S production.

MATERIALS AND METHODS

Microorganism and growth conditions. Type strains of oral Veillonella, namely, V. atypica ATCC 17744, V. dispar ATCC 17748, and V. parvula ATCC 10740, were used throughout the present study. These bacteria were precultured in a complex medium containing 0.5% tryptone (Becton, Dickinson and Company, Sparks, MD), 0.3% yeast extracts (Becton, Dickinson and Company), 1.8% sodium lactate, and 40 mM potassium phosphate buffer (PPB) (pH 7) (TYL medium) in an anaerobic chamber (Hirasawa Works, Tokyo, Japan) (NHC type [N$_2$, 80%; CO$_2$, 10%; H$_2$, 10%]) at 37°C.

Bacterial growth and H$_2$S production. Veillonella species grown to the logarithmic phase were transferred into new TYL medium in the presence or absence of 1 mM L-cysteine at pH 7. Bacterial growth was monitored for 48 h by the optical density (OD) at 660 nm. At 48 h, concentrations of H$_2$S in supernatants of culture media were measured using the methylene blue method as described below.

Effects of pH and lactate on H$_2$S production from L-cysteine by resting cells. Bacteria were grown to the logarithmic growth phase at pH 7 as described above and harvested by centrifugation. Bacterial cells were washed twice with 2 mM potassium phosphate buffer (PPB) (pH 7) containing 75 mM KCl, 75 mM NaCl, and 2 mM MgCl$_2$ and suspended in the same buffer solution. The concentration of cell suspension was adjusted according to the OD at 660 nm. Then, 0.15-mL cell suspensions (OD at 660 nm = 20 [approximately corresponding to 1.2 to 1.7 mg dry weight of cells]) were incubated at 37°C with 1 mM L-cysteine at pH 5, 6, and 7. In addition, the cell suspension was incubated at 37°C with 1 mM cysteine at pH 5 or 7 in the presence or absence of 0.01 to 100 mM sodium lactate, sodium acetate, or glucose. The concentration of H$_2$S in the cell suspension was measured using the methylene blue method as described below.

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H₂S and other metabolic products from l-cysteine by cell extracts and cell membrane. Bacteria were harvested and washed as described above for the cell suspension experiment and stored as cell pellets at −20°C. After thawing, the cell pellets were suspended in 2 mM PPB (pH 7.0) containing 75 mM KCl, 75 mM NaCl, and 2 mM MgCl₂ and oscillated anaerobically by ultrasonication (Insonator 201M; Kubota, Tokyo, Japan) (2 A, 190 W, 4°C, 7 min) as described previously (12). Cell debris and unbroken cells were removed by centrifugation (17,700 g, 4°C, 7 min), and the resultant cell extract was used for the detection of enzymatic activity. Part of the cell extract was centrifuged further (17,700 g, 4°C, 7 min), and the precipitate was washed twice with the buffer and centrifuged again. The resultant precipitate was suspended in the buffer and designated the cell membrane fraction. The reaction mixture (1.5 ml) containing 1 mM l-cysteine, 0 to 10 mM sodium lactate, and the cell extract (0.1 ml) or cell membrane fraction (0.1 ml) was incubated at 37°C in 50 mM sodium phosphate buffer (pH 7.0) containing 75 mM KCl, 75 mM NaCl, and 2 mM MgCl₂ and oscillated at 20°C. After thawing, the cell pellets were suspended in 2 mM PPB (pH 7.0) containing 75 mM KCl, 75 mM NaCl, and 2 mM MgCl₂ and oscillated anaerobically by ultrasonication (Insonator 201M; Kubota, Tokyo, Japan) (2 A, 190 W, 4°C, 7 min) as described previously (12). Cell debris and unbroken cells were removed by centrifugation (17,700 g, 4°C, 7 min), and the resultant cell extract was used for the detection of enzymatic activity. Part of the cell extract was centrifuged further (17,700 g, 4°C, 7 min), and the precipitate was washed twice with the buffer and centrifuged again. The resultant precipitate was suspended in the buffer and designated the cell membrane fraction. The reaction mixture (1.5 ml) containing 1 mM l-cysteine, 0 to 10 mM sodium lactate, and the cell extract (0.1 ml) or cell membrane fraction (0.1 ml) was incubated at 37°C in 50 mM PPB (pH 5 or 7).

The production of H₂S was measured using the methylene blue method as described below. Pyruvate and ammonia were quantified by the enzymatic method (13) and using an ammonia meter (Amichek AA4120 meter; Arkley, Kyoto, Japan), respectively. Serine and lanthionine were quantified with capillary electrophoresis and a time-of-flight mass spectrometer (CE-TOFMS) as described below. Protein concentrations in cell extracts and cell membrane fractions were measured by the dye method (protein assay dye reagent concentrate; Bio-Rad).

H₂S measurement. The methylene blue method (14, 15) was used with minor modification. Reaction mixtures were centrifuged (7,740 × g, room temperature, 3 min), and supernatants (3 ml) were mixed with 0.205 ml di-methyl p-phenylene diamine solution and then 0.03 ml of 27.7 mM FeCl₃ solution. After 1 min, the mixture was mixed with 0.82 ml of 3 M (NH₄)₂HPO₄ solution. After 15 min at room temperature, the concentration of H₂S was estimated photometrically at 665 nm. Dimethyl p-phenylene diamine solution was prepared as follows: 2 g dimethyl p-phenylene diamine was dissolved in 12 N HCl (total volume, 10 ml), and 2 ml of the solution was mixed with 48 ml deionized H₂O and 50 ml sulfuric acid.

Serine and lanthionine measurement with CE-TOFMS. The reaction mixture described above was pretreated as described previously (16–19). The reaction mixture (75 μl) described above was mixed with 675 μl methanol containing internal standards (Internal standard solution-1; Human Metabolome Technologies), 750 μl chloroform, and 300 μl Milli-Q water, mixed using a vortex device for 30 s, and centrifuged at 4,600 × g and 4°C for 5 min. The aqueous layer was distributed into 3 ultraliter filters (Ultratrace-MC 5000NMWL UFC3 LCCNB; Millipore, Billerica, MA) (250 μl each) and centrifuged at 9,100 × g and 0°C overnight. The filtrate was dried for 6 to 9 h, suspended in 30 μl Milli-Q water containing internal standards (Internal standard solution-3; Human Metabolome Technologies), and stored at −80°C until analysis. Internal standard solution-1 contains camphor-10-sulfonic acid to calibrate the quantification of MS. Internal standard solution-3 contains trimesic acid and 3-hydroxynaphthalene-2,7-disulfonic acid to calibrate the retention time for CE.

CE-MS was carried out by CE using a G1600AX system (Agilent Technologies, Waldbronn, Germany) equipped with a time-of-flight mass spectrometer (TOFMS) (G1969A; Agilent Technologies). Separation and detection of metabolites were performed as described previously (16, 18, 19, 20). All standard metabolites and chemicals used were of analytical or reagent grade. The obtained data were processed using calculating software (MassHunter Workstation Software Qualitative Analysis; Agilent Technologies) and data obtained from standard metabolite solutions.

**Statistical analysis.** The paired t test and Dunnett test were used for statistical analysis. Values of P < 0.05 were considered significant.

### RESULTS

**Growth and H₂S production in the presence of l-cysteine.** The supplementation of culture media with 1 mM l-cysteine significantly increased H₂S production by *Veillonella* species during growth (Table 1). However, l-cysteine did not stimulate growth.

#### TABLE 1  Bacterial growth and H₂S production with and without l-cysteine

<table>
<thead>
<tr>
<th>Species</th>
<th>Without 1 mM l-cysteine</th>
<th>With 1 mM l-cysteine</th>
<th>H₂S production (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. atypica</td>
<td>1.20 ± 0.03</td>
<td>1.29 ± 0.01</td>
<td>166 ± 19.7</td>
</tr>
<tr>
<td>V. dispar</td>
<td>0.75 ± 0.01</td>
<td>1.08 ± 0.15</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>V. parvula</td>
<td>1.53 ± 0.11</td>
<td>1.14 ± 0.05</td>
<td>187 ± 20.6</td>
</tr>
</tbody>
</table>

a Data represent OD at 660 nm after 48 h.
b Data represent μM in the media. *, significant difference from the results obtained without l-cysteine (P < 0.05) by paired t test.

FIG 1  Effects of pH and lactate on H₂S production from l-cysteine for 3 h by oral *Veillonella*. Insets show relative levels of H₂S production at pH 5, 6, and 7 in the absence of lactate. The mean values from three independent experiments are shown. Vertical bars show standard deviations.
Effects of pH and lactate on H$_2$S production from l-cysteine by resting cells. The resting cells of Veillonella species produced H$_2$S from l-cysteine, and the amount of H$_2$S increased with time for 3 h, so the amount of H$_2$S produced over 3 h was determined as follows.

H$_2$S production was higher at pH 6 and 7 than at pH 5 without lactate (insets in Fig. 1). With the addition of lactate, H$_2$S production increased markedly and peaked (4.4- to 8.7-fold) in the presence of 100 mM lactate at pH 7, while H$_2$S production at pH 5 peaked (4.5- to 23.7-fold) in the presence of 10 mM lactate but decreased in the presence of 100 mM lactate (Fig. 1). Acetate or glucose had no effect (data not shown).

H$_2$S and other metabolic products from l-cysteine by cell extracts and cell membrane. Addition of 1 mM l-cysteine to the cell extracts resulted in H$_2$S production, and the amount of H$_2$S increased with time for 60 min, so the amounts of H$_2$S and other metabolic products over 60 min were determined as follows. No H$_2$S was produced by the cell membrane (data not shown).

H$_2$S-producing activity without lactate was significantly higher at pH 7 than at pH 5 in the cell extracts of _V. atypica_ (Table 2), while the levels of activity were similar at pH 7 and 5 in the other Veillonella strains. Lactate did not increase H$_2$S production by the cell extracts of any of the Veillonella species. Accompanied with H$_2$S production, ammonia was produced by all the Veillonella species. Pyruvate was produced only by _V. atypica_ and _V. parvula_. Serine was also produced only by _V. atypica_ and _V. parvula_. No lanthionine was produced by any species (Table 3).

**DISCUSSION**

Although _Veillonella_ has long been known to produce H$_2$S (8, 10, 11), the present report is the first to show that oral _Veillonella_ species have metabolic activity that produces H$_2$S from l-cysteine (Table 1, Fig. 1). l-Cysteine is detected in both saliva (21) and serum (22). In addition, various peptides containing cysteine are available in these fluids and supplied by desquamation of the epithelium. In particular, keratin, as the major protein in desquamation, containing a number of l-cysteine molecules as a form of cystine, may serve as a source of l-cysteine in the oral cavity. In spite of l-cysteine utilization, there was no significant increase in growth in the presence of additional l-cysteine (Table 1), suggesting that l-cysteine is not utilized as a main energy source.

H$_2$S production was increased by lactate in the cell suspension (Fig. 1) but not in the cell extracts (Table 2), suggesting that lactate activates not the enzyme itself but the process prior to l-cysteine degradation, such as the incorporation of l-cysteine across the cell membrane. It was previously reported that membrane vesicles prepared from _Veillonella alcalescens_ possessed uptake activity for amino acids (l-glutamate and l-serine) coupled with an electron transport system in which lactate can participate as an electron donor (23). _Veillonella_ species used in the present study may possess a similar system. In addition, _Veillonella_ species utilize lactate as a main energy source (17, 24), and the generated ATP may thus energize an ATP-dependent transport system for amino acids such as the ATP-binding cassette transporter, which has been found in a wide range of bacteria (25).

The concentration of lactic acid in the tongue coating is known to reach 6.7 to 7.8 mM after a sucrose rinse (26, 27) due to the dominance of lactate-producing bacteria such as streptococci (5) and probably results in acidification of the tongue coating. Thus, in the tongue coating, changes in both pH and the lactate concentration may influence H$_2$S production by _Veillonella_. According to the results of the present study, environmental acidification by lactate production can decrease H$_2$S production whereas the accumulation of lactate can, in contrast, increase H$_2$S production. It is known that food intake reduces the oral malodor level rapidly while, after eating, oral malodor increases gradually (28). The rapid reduction of malodor is thought to be mainly due to the removal of odorous compounds by mastication and salivary secretion but could be partly due to environmental acidification. The consequent increase of malodor is possibly attributable to H$_2$S production by _Veillonella_ which is stimulated by lactate produced and retained in the tongue coating.

Glucose did not increase H$_2$S production by _Veillonella_, indicating that the enhancement of H$_2$S production by _Veillonella_ requires coexistence with lactate-producing bacteria such as strep-

**TABLE 2** H$_2$S production from l-cysteine by cell extracts in the presence or absence of lactate for 60 min

<table>
<thead>
<tr>
<th>Species</th>
<th>Lactate concn (mM)</th>
<th>H$_2$S production (mM) at pH (mean ± SD)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. atypica</em></td>
<td>None</td>
<td>100 ± 10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>92 ± 11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>83 ± 10</td>
</tr>
<tr>
<td><em>V. dispar</em></td>
<td>None</td>
<td>100 ± 11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>114 ± 41</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>117 ± 63</td>
</tr>
<tr>
<td><em>V. parvula</em></td>
<td>None</td>
<td>100 ± 8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>112 ± 27</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>135 ± 16</td>
</tr>
</tbody>
</table>

$^a$ Data represent mean values ± standard deviations from three independent experiments. The H$_2$S production from l-cysteine without lactate at pH 7 was regarded as 100. *, significant difference from pH 7 ($P < 0.05$) by paired t test.

**TABLE 3** Production of H$_2$S and other metabolic products from l-cysteine by cell extracts at pH 7.0 for 60 min

<table>
<thead>
<tr>
<th>Species</th>
<th>H$_2$S</th>
<th>Ammonia</th>
<th>Pyruvate</th>
<th>Serine</th>
<th>Lanthionine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. atypica</em></td>
<td>11.9 ± 1.8</td>
<td>16.6 ± 14.2</td>
<td>11.4 ± 13.0</td>
<td>4.4 ± 7.1</td>
<td>ND</td>
</tr>
<tr>
<td><em>V. dispar</em></td>
<td>2.8 ± 2.2</td>
<td>4.8 ± 1.3</td>
<td>0.0 ± 1.0</td>
<td>0.0 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td><em>V. parvula</em></td>
<td>9.3 ± 3.0</td>
<td>3.0 ± 2.7</td>
<td>2.6 ± 5.1</td>
<td>0.6 ± 1.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Data represent the mean values ± standard deviations of the amount of each of the metabolites (nmol per mg protein) from three independent experiments. ND, not detected.
H$_2$S Production by Veillonella and pH/Lactate

tococci. Previous studies have shown symbiosis between Veillonella and Streptococcus in dental plaque, with Streptococcus producing lactate and acting as an energy source for Veillonella (29, 30). In addition, coexistence with Veillonella induced the expression in Streptococcus gordoni of α-amylase, which enables Streptococcus to degrade starch to oligosaccharides and consequently metabolize them into lactate that can be used by Veillonella (31). The present study suggested an additional finding of symbiosis: that lactate produced by saccharolytic bacteria stimulates amino acid metabolism, including t-cysteine and, subsequently, H$_2$S production by Veillonella.

Cell extracts showed enzymatic activity resulting in production of H$_2$S from t-cysteine, while the cell membrane fraction displayed no such activity, indicating that the H$_2$S-producing enzyme is located in the cytoplasm. Among oral bacteria, Fusobacterium species (32–35) have been reported to possess cystathionine γ-lyase (EC 4.4.1.1) (which cleaves L-cysteine to pyruvate, ammonia, and H$_2$S at a ratio of 1:1:1), cystathionine β-synthase lyase (EC 4.2.1.22) (which cleaves L-cysteine to serine and H$_2$S at a ratio of 1:1), and cysteine lyase (EC 4.4.1.10) (which catalyzes the synthesis of lanthionine and H$_2$S at a ratio of 1:1 from 2 cysteines) (Fig. 2). Treponema denticola (36) and Streptococcus anginosus (37) also have been reported to possess cystathionine γ-lyase. The observation that ammonia was produced along with H$_2$S by all the species (Table 3) suggests that oral Veillonella possess cystathionine γ-lyase. However, pyruvate was not produced by V. dispar, suggesting that pyruvate might be further metabolized in this bacterium. The production of serine by V. atypica and V. parvula indicates that these species also possess cystathionine β-synthase and convert a part of t-cysteine to H$_2$S and serine. Lanthionine was not detected, indicating that oral Veillonella do not have cysteine lyase. Throughout these experiments, however, metabolite quantification did not satisfy the stoichiometry, and thus further study is needed to clarify the enzymes responsible for H$_2$S production.

In conclusion, the present study elucidated that oral Veillonella species produce H$_2$S from t-cysteine by a process in which various enzymes, including cystathionine β-synthase and cystathionine γ-lyase, are involved. Moreover, H$_2$S production can be influenced by oral environmental factors, namely, pH and lactate. Since oral Veillonella species predominate as H$_2$S-producing bacteria in the tongue coating of orally malodorous patients without periodontitis (9), controlling the environmental factors of pH and lactate might provide a practical method to prevent oral malodor.

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