Hydrogen Sulfide Production by Oral *Veillonella*: Effect of pH and Lactate

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Running head: H₂S production by *Veillonella* and pH/lactate

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ABSTRACT

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 – 7 and lower at pH 5. The presence of lactate markedly increased H$_2$S production by resting cells (4.5 – 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 $\gamma$-lyase (EC 4.4.1.1). Serine was also produced in cell extracts of *V. atypica* and *V. parvula*, suggesting the involvement of cystathionine $\beta$-synthase lyase (EC 4.2.1.22) in these strains. This study indicates that *Veillonella* produce H$_2$S from L-cysteine and their H$_2$S production can be regulated by oral environmental factors, pH and lactate.
INTRODUCTION

Oral 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 bacteria 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 (H₂S), one of the major components of oral malodor (7, 8). In the previous study (9), we focused on oral malodor in patients without oral diseases such as periodontitis or caries, and found that the predominant H₂S-producing bacteria were not periodontitis-related, but 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 H₂S (1, 8, 10, 11), the metabolic properties of H₂S 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 H₂S production by Veillonella.

In the present study, therefore, we investigated the metabolic properties of the H₂S
production of oral Veillonella species using growing cells, resting cells and cell extracts of
Veillonella atypica, Veillonella dispar and Veillonella parvula, and the effects of oral
environmental factors, pH and lactate on H₂S production.

MATERIALS AND METHODS

Microorganism and growth conditions

Type strains of oral Veillonella, V. atypica ATCC17744, V. dispar ATCC17748 and V.
parvula ATCC10740, were used throughout the present study. These bacteria were
pre-cultured in a complex medium containing 0.5% tryptone (Becton, Dickinson and Company, Sparks, MD, USA), 0.3% yeast extracts (Becton, Dickinson and Company) and 1.8% sodium lactate in 40 mM potassium phosphate buffer (pH 7) (TYL medium) in an anaerobic chamber (N₂, 80%; CO₂, 10%; H₂, 10%; NHC-type; Hirasawa Works, Tokyo, Japan) at 37°C.

Bacterial growth and H₂S 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 optical density (OD) at 660 nm. At 48 h, concentrations of H₂S in supernatants of culture media were measured using the methylene blue method as described below.

Effects of pH and lactate on H₂S 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 MgCl₂, and suspended in the same buffer solution. The concentration of cell suspension was adjusted by OD at 660 nm. Then, 0.15 mL cell suspensions (OD at 660 nm = 20; approximately corresponding to 1.2-1.7 mg dry weight of cells) was 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 0.01-100 mM sodium lactate, sodium acetate or glucose. The concentration of H₂S in the cell suspension was measured using the methylene blue method as described below.

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 (2A, 190 W, 4°C, 7 min, Insonator 201M; Kubota, Tokyo, Japan) as described previously (12). Cell debris and unbroken cells were removed by centrifugation (17700xg, 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 (17700xg, 4°C, 1 h) and the precipitate was washed twice with the buffer and centrifuged again. The resultant precipitate was suspended in the buffer and designated as the cell membrane fraction. The reaction mixture (1.5 mL) containing 1 mM L-cysteine, 0-10 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 meter, AA4120; 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 concentration in cell extracts
and cell membrane fraction was measured by the dye-method (Protein assay dye reagent concentrate, Bio-rad, CA, USA).

**H₂S measurement**

The methylene blue method (14, 15) was used with minor modification. Reaction mixtures were centrifuged (7740xg, 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 12N HCl (total volume of 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, vortexed for 30 sec, and centrifuged at 4600 x g and 4°C for 5 min. The aqueous layer was distributed into 3 ultrafilter sets (250 µL each) (Ultrafree-MC 5000NMWL UFC3 LCCNB; Millipore, Billerica, MA, USA) and centrifuged at 9100 x g and 0°C overnight. The filtrate was dried for 6-9 hrs, suspended in 50 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 (G1600AX; 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. Obtained data were processed by calculating software (MassHunter Workstation Software Qualitative Analysis; Agilent Technologies), using 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.

**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 hr, so the amount of H$_2$S produced over 3 hr was shown in the following experiments.

H$_2$S production was higher at pH 6 and 7 than pH 5 without lactate (insets in Fig. 1). With the addition of lactate, H$_2$S production increased markedly and peaked (4.4 – 8.7-fold) in the presence of 100 mM lactate at pH 7, while H$_2$S production at pH 5 peaked (4.5 – 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 \( H_2S \) production, and the amount of \( H_2S \) increased with time for 60 min, so the amounts of \( H_2S \) and other metabolic products over 60 min were shown in the following experiments. No \( H_2S \) was produced by the cell membrane (data not shown).

\( H_2S \) 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 activity was similar at pH 7 and 5 in the other \( Veillonella \) strains. Lactate did not increase \( H_2S \) production by the cell extracts of all the \( Veillonella \) species. Accompanied with \( H_2S \) 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 \( H_2S \) (8, 10, 11), the present study is
the first to show that oral *Veillonella* species have a metabolic activity to produce 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 reported that membrane vesicles prepared from *Veillonella alcalescence* 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 also possess a similar system. In addition, Veillonella species utilize lactate as a main energy source (17, 24), and thus the generated ATP may 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 – 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 lactate concentration may influence H$_2$S production by Veillonella. According to the present study, environmental acidification by lactate production can decrease H$_2$S production, while the accumulation of lactate can reversely 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 attributed to H₂S production by *Veillonella*, which is stimulated by lactate produced and retained in the tongue coating.

Glucose did not increase H₂S production by *Veillonella*, indicating that the enhancement of H₂S production by *Veillonella* requires the coexistence of lactate-producing bacteria such as streptococci. Previous studies have shown symbiosis between *Veillonella* and *Streptococcus* in dental plaque, with *Streptococcus* producing lactate and acting as an energy source of *Veillonella* (29, 30). In addition, coexistence with *Veillonella* induced the expression of α-amylase in *Streptococcus gordonii*, which enables *Streptococcus* to degrade starch to oligosaccharides and consequently metabolize them into lactate that can be used by *Veillonella* (31). The present study suggests an additional finding of symbiosis that lactate produced by saccharolytic bacteria stimulates amino acid metabolism including L-cysteine and subsequently H₂S production by *Veillonella*.

Cell extracts showed enzymatic activity to produce H₂S from L-cysteine, while the cell membrane fraction displayed no such activity, indicating that the H₂S-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 H₂S at a ratio of 1:1:1), cystathionine β-synthase lyase (EC 4.2.1.22; which cleaves L-cysteine to serine and H₂S at a ratio of 1:1), and cysteine lyase (EC 4.4.1.10; which catalyzes the synthesis of lanthionine and H₂S at a ratio of 1:1 from 2 cysteine) (Fig. 3). *Treponema denticola* (36) and *Streptococcus anginosus* (37) also have been reported to possess cystathionine γ-lyase. The observation that ammonia was produced along with H₂S production 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 L-cysteine to H₂S 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 H₂S production.

In conclusion, the present study elucidated that oral *Veillonella* species produce H₂S from L-cysteine, in which *various enzymes including* cystathionine β-synthase and cystathionine γ-lyase are involved. Moreover, H₂S production can be influenced by oral environmental factors, pH and lactate. Since oral *Veillonella* species predominate as H₂S-producing bacteria in the tongue coating of oral malodorous patients without periodontitis (9), controlling environmental factors, pH and lactate might provide a practical method to prevent oral malodor.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Young Scientists B (No. 23792498 to JW) and Grants-in-Aid for Scientific Research B (No. 22390399 to NT) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.
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characterization of the L-cysteine desulphhydrase gene of Fusobacterium nucleatum.


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Figure legends

Fig. 1. Effects of pH and lactate on H₂S production from L-cysteine for 3 hr by oral Veillonella. Insets show relative 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.

Fig. 2. The major metabolic pathways for H₂S production from L-cysteine
Table 1. Bacterial growth and H₂S production with and without L-cysteine

<table>
<thead>
<tr>
<th></th>
<th>V. atypica</th>
<th>V. dispar</th>
<th>V. parvula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial growth with;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1.20 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.01</td>
<td>1.53 ± 0.11</td>
</tr>
<tr>
<td>1 mM L-cysteine</td>
<td>1.29 ± 0.01</td>
<td>1.08 ± 0.15</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>H₂S production with;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>166 ± 19.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8 ± 0.7</td>
<td>187 ± 20.6</td>
</tr>
<tr>
<td>1 mM L-cysteine</td>
<td>224 ± 13.8*</td>
<td>117 ± 38.8*</td>
<td>670 ± 186*</td>
</tr>
</tbody>
</table>

The mean values ± standard deviations from three independent experiments were shown.

<sup>a</sup>, OD at 660 nm after 48 h.

<sup>b</sup>, μM in the media.

<sup>*</sup>, Significant difference from without L-cysteine (p < 0.05) by paired t-test.
Table 2. H₂S production from L-cysteine by cell extracts in the presence or absence of lactate for 60 min.

<table>
<thead>
<tr>
<th></th>
<th>Lactate</th>
<th>pH 7</th>
<th>pH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. atypica</em></td>
<td>none</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40 ± 15&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>92 ± 11</td>
<td>43 ± 16&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>83 ± 10</td>
<td>43 ± 11&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>V. dispar</em></td>
<td>none</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 29</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>114 ± 41</td>
<td>121 ± 64</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>117 ± 63</td>
<td>117 ± 58</td>
</tr>
<tr>
<td><em>V. parvula</em></td>
<td>none</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87 ± 40</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>112 ± 27</td>
<td>77 ± 50</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>135 ± 16</td>
<td>62 ± 32</td>
</tr>
</tbody>
</table>

The mean values ± standard deviations from three independent experiments were shown.

<sup>a</sup> The H₂S production from L-cysteine without lactate at pH 7 was regarded as 100.

<sup>*</sup> Significant difference from pH 7 (p<0.05) by paired t-test.
Table 3. H$_2$S and other metabolic products from L-cysteine by cell extracts at pH 7.0 for 60 min.

<table>
<thead>
<tr>
<th></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$^a$</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>

The mean values ± standard deviations from three independent experiments were shown.

$^a$The increase of each metabolites (nmole per mg protein).

nd, not detected.
Fig. 1

H$_2$S production (µmol/mg dry weight)

% H$_2$S production without lactate (100 at pH 7)

Lactate (mM)

V. parvula

V. dispar

V. atypica
\[
\text{Cysteine} \xrightarrow{\text{cystathionine } \gamma\text{-lyase}} \text{H}_2\text{S} + \text{NH}_3 + \text{Pyruvate} \\
\text{Cysteine} + \text{Cysteine} \xrightarrow{\text{cystathionine } \beta\text{-synthase}} \text{H}_2\text{S} + \text{Serine} \\
\text{Cysteine} + \text{Cysteine} \xrightarrow{\text{cysteine lyase}} \text{H}_2\text{S} + \text{Lanthionine}
\]