NOTE

Estimation of bacterial hydrogen sulfide production *in vitro*

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Oral bacterial hydrogen sulfide (H_2S) production was estimated comparing two different colorimetric methods in microtiter plate format. High H_2S production was seen for *Fusobacterium* spp., *Treponema denticola*, and *Prevotella tannerae*, associated with periodontal disease. The production differed between the methods indicating that H_2S production may follow different pathways.

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Periodontal disease is believed to be associated with an anaerobic and proteolytic bacterial metabolism but the pathogenesis is still largely unknown. Hydrogen sulfide (H_2S) is a toxic, bacterial waste product in the subgingival pocket and up to 1.9 mM H_2S has been detected in gingival crevicular fluid (1, 2). Due to its proinflammatory properties, it has been suggested that H_2S may participate in the bacteria-induced inflammatory response in the periodontal diseases (3–6).

Previous studies have reported bacterial H_2S production from various species, for example, *Fusobacterium* spp., by the degradation of cysteine (7), homocysteine (8), and glutathione (9) and from *Parvimonas micra*, *Tannerella forsythia* and *Filifactor alocis* (1). More research is, however, needed to elucidate the rate and the amount of H_2S produced by various species/strains under various conditions both *in vitro* and *in vivo*.

The H_2S-producing capacity is commonly tested by blackening of lead acetate paper (10) or with gas chromatography (1, 11) and sensors (12–14). These methods are either rough or require complex equipment and are therefore expensive. Simple chair-side methods for semi-quantification of bacterial H_2S, which could further facilitate the investigation of H_2S production and presence, are lacking.

The aim of the present study is to examine oral bacterial H_2S production *in vitro* comparing two colorimetric methods in microtiter plate format.

The bacterial species tested for H_2S-producing capacity are given in Table 1. The species were grown on appropriate agar plates under optimal conditions.

The bismuth sulfide (BS) method introduced by Yoshida et al. (8) was modified to using a 5 mM concentration of bismuth(III)chloride. Bacteria were diluted in peptone solution to 10^9 cells/mL. Aliquots (100 µl) of the bacterial suspension were mixed with an equal amount of newly prepared bismuth solution (0.4 M triethanolamine-HCl, pH 8.0; 10 mM bismuth(III)chloride; 20 µM pyridoxal 5-phosphate monohydrate, 20 mM EDTA and 40 mM L-cysteine) in microtiter plates. In the presence of H_2S, black BS is precipitated. Three technical staff members measured the precipitation by scoring the color of the well using a visual scale, from no color production (0) to maximum black color production (5) every hour between 0 and 7 and also after 24 h (Fig. 1a). In addition to the visual scale (median), BS precipitation was determined (mean) by measuring the optical density (OD_{405}).

For the methylene blue (MB) method, adapted after Cline (15), bacterial colonies from the agar plates were incubated in broth with 20 mM L-cysteine until approximately 10^9 cells/mL were obtained. Thereafter, 10 µl was transferred to 72 µl of distilled water (pH 9.6 + 0.1 mM diethylenetriaminepentaacetic acid) (16) in a microtiter well. A volume of 18 µl of a solution (17.1 mM *N*, *N*-dimethyl-*p*-phenylenediamine sulfate salt and 37.0 mM $FeCl_3$ in 6 M HCl) was immediately added (17). The MB

Table 1. Bacterial hydrogen sulfide (H_2S) production from cysteine measured with two colorimetric methods in microtiter plate format, recorded as black bismuth sulfide (BS) precipitation and methylene blue (MB) formation

Species	Strain	H_2S production[a] BS method	MB method
Aggregatibacter actinomycetemcomitans	ATCC[b] 43718	+	−
Actinomyces naeslundii	ATCC 12104	−	−
Actinomyces odontolyticus	ATCC 17929	−	−
Actinomyces oris	ATCC 15987	−	−
Bifidobacterium dentium	ATCC 11863	−	−
Eikenella corrodens	ATCC 23834	+	−
Enterococcus faecalis	ATCC 19433	+	−
Filifactor alocis	ATCC 35896	−	−
Fusobacterium necrophorum	ATCC 51357	+ + + +	+ + + +
Fusobacterium necrophorum	CCUG[c] 48192	+ + + +	+ + + +
Fusobacterium nucleatum	ATCC 10953	+ + + +	+ + + +
Fusobacterium periodonticum	ATCC 33693	+ + + +	+ + +
Lactobacillus casei	CCUG 31610	−	−
Lactobacillus gasseri	ATCC 33323	−	−
Lactobacillus rhamnosus	ATCC 7469	−	−
Lactobacillus salivarius	CCUG 55845	−	−
Parvimonas micra	ATCC 33270	+ + +	+
Porphyromonas endodontalis	OMGS[d] 1205	−	+ +
Porphyromonas gingivalis 381F	OMGS 2860	+	+ + +
Porphyromonas gingivalis W83	OMGS 197	+	+
Prevotella intermedia	ATCC 25611	+	−
Prevotella nigrescens	ATCC 33563	−	−
Prevotella tannerae	ATCC 51259	+ +	+ + +
Rothia dentocariosa	CCUG 17835	+	−
Staphylococcus aureus	OMGS 3871	−	−
Streptococcus anginosus	ATCC 12395	+ +	−
Streptococcus gordonii	ATCC 33399	−	−
Streptococcus intermedius	ATCC 27335	−	−
Streptococcus oralis	ATCC 35037	−	−
Streptococcus mitis	ATCC 49456	−	−
Streptococcus mutans	ATCC 25175	−	−
Streptococcus salivarius	ATCC 7073	−	−
Streptococcus sanguinis	ATCC 10556	−	−
Streptococcus sobrinus	CCUG 27507	−	−
Tannerella forsythia	ATCC 43037	−	−
Treponema denticola	OMGS 3271[e]	+ + + +	+ + + +
Veillonella parvula	ATCC 10790	+	−

[a]Visual color change, scored from no color production (−) to maximum black or blue color production (+ + + +) by three individuals. The results of the bismuth method are shown after 7 h of incubation in the bismuth solution. Data shown are median values of triplicate wells for three repetitions of the experiment.
[b]American Type Culture Collection.
[c]Culture Collection University of Gothenburg.
[d]Oral Microbiology Gothenburg Sweden.
[e]Originally received from Dr. R. Ellen, University of Toronto, Toronto, Canada.

method measures the H_2S produced during bacterial growth in the broth and the formation was measured 30 min after the addition of the solution (17) (Fig. 1b).

Similar to the BS method, visual-scale estimations (median) and optical density (OD_{668}) measurements (mean) were performed.
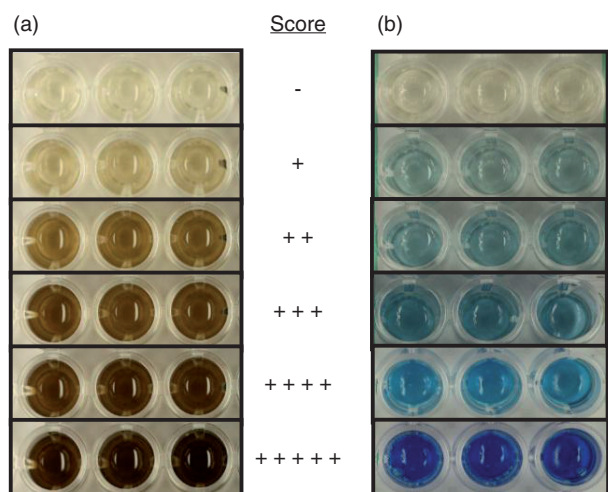
Fig. 1. (a) The BS method. The production of H$_2$S was estimated by black BS precipitation and recorded as no color production (−; score 0) to maximum black color production (+ + + +; score 5). (b) The methylene blue (MB) method. The production of MB in the presence of H$_2$S was estimated from no color production (−; score 0) to maximum blue color production (+ + + + ; score 5).

To test the sensitivity of the methods, different concentrations of sodium hydrosulfide (NaHS) were mixed with the BS and MB solutions. In both cases, a linear correlation between the color intensity and the HS$^-$/S^{2-} ion concentration was found with the spectrophotometric and the visual determination (Fig. 2). The visual detection limit for H$_2$S was 0.6 mM for the BS method and 2 mM for the MB method.

The most rapid H$_2$S production was seen for *Fusobacterium* spp. reaching the maximum color production (Fig. 3). *Treponema denticola*, *P. micra*, and *Streptococcus anginosus* also showed changes in OD, while *Lactobacillus casei* and both strains of *Porphyromonas gingivalis* failed to do so. Also, the highest production was seen for *Fusobacterium* spp. (Table 1). *T. denticola* showed a clear color change with both methods but at a slower rate. Interestingly, *Prevotella tannerae* had a high capacity to produce H$_2$S reaching a concentration of approximately 3 mM according to both methods. To our knowledge, this strain has not previously been tested for its H$_2$S-producing capacity. Also, *P. micra* and *P. gingivalis* produced H$_2$S but the color production differed between the BS and MB methods and between the two *P. gingivalis* strains tested. Although *P. gingivalis* W83 is believed to be more virulent than 381F (18, 19), the latter showed higher H$_2$S production in broth (MB method). This could result from more proteins being available for degradation in the broth than in the BS solution. A preference for proteins before amino acids as source of nutrients (20) may also be true for *P. endodontalis*. The results indicate that H$_2$S production may follow different pathways in the two methods.
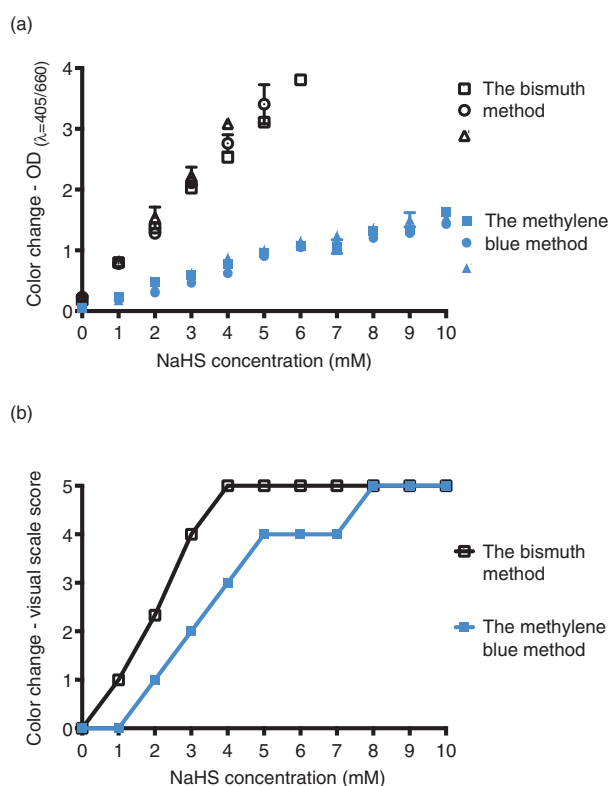

Fig. 2. NaHS was tested with two colorimetric tests: the BS method (black) and the methylene blue (MB) method (blue). The color production for known concentrations of NaHS was a) determined (mean) with optical density analysis ($\lambda = 405$ nm for the bismuth method and $\lambda = 668$ nm for the MB method), b) scored on a visual scale by three individuals (median). Data shown are individual values for triplicates of the test.

Both methods, based on BS precipitation and MB formation, respectively, had a high reproducibility, reliability, and simplicity. The highest rate to maximal detectable production of H$_2$S was found for *Fusobacterium* spp. Different findings from the two methods for some bacteria may reflect different pathways used for H$_2$S production and, therefore, the BS and MB methods may complement one another. The BS method was more sensitive than the MB method and may be suitable for *in vivo* estimation of H$_2$S production, using, for example, plaque samples from bacterial infections, such as in the periodontal pockets and other anaerobic infection sites. The production of H$_2$S is complex and needs more attention in future studies to enhance the knowledge of the mechanisms involved in H$_2$S production and its impact *in vivo*.

Conflict of interest and funding
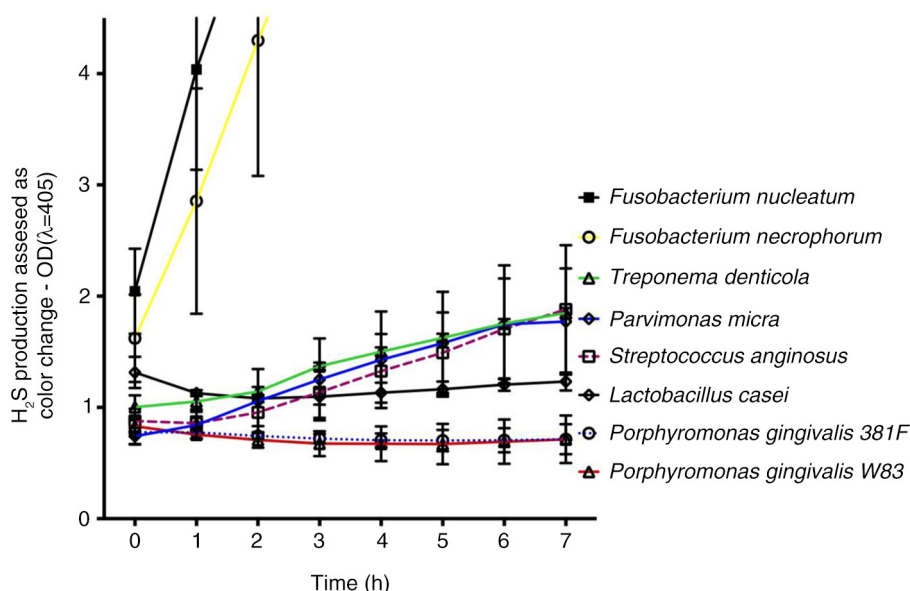
Fig. 3. Bacterial H₂S production was determined by BS precipitation and optical density (OD$_{405}$) measurements up to 7 h at different time points. The bacteria were analyzed at a concentration of approximately 5×10^8 cells/mL. Data shown are mean values for triplicate wells for three repetitions of the experiment.

Bengtsson for visual scale scoring. There is no conflict of interest in the present study for any of the authors.

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