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Differences in the βC-S lyase activities of viridans group streptococci☆

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Abstract

βC-S Lyase catalyzes the α,β-elimination of L-cysteine to hydrogen sulfide, which is one of the main causes of oral malodor and is highly toxic to mammalian cells. We evaluated the capacity of six species of oral streptococci to produce hydrogen sulfide. The crude enzyme extract from *Streptococcus anginosus* had the greatest capacity. However, comparative analysis of amino acid sequences did not detect any meaningful differences in the *S. anginosus* βC-S lyase. The capacity of *S. anginosus* purified βC-S lyase to degrade L-cysteine was also extremely high, while its capacity to degrade L-cystathionine was unremarkable. These findings suggest that the extremely high capacity of *S. anginosus* to produce hydrogen sulfide is due to the unique characteristic of βC-S lyase from that organism.

Keywords: βC-S lyase; Streptococci; Hydrogen sulfide; Pyridoxal 5′-phosphate-dependent enzyme; Cysteine; Cystathionine

βC-S Lyase is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the α,β-elimination of L-cysteine into hydrogen sulfide, pyruvate, and ammonia. Along with methyl mercaptan, dimethyl sulfide, and dimethyl disulfide, hydrogen sulfide is one of the predominant volatile sulfur compounds responsible for oral malodor. Hydrogen sulfide is also highly toxic for mammalian cells [1] and induces the modification and release of hemoglobin in erythrocytes [2]. Many investigations have focused on Gram-negative periodontopathogenic bacteria as the main organisms capable of producing hydrogen sulfide [3–5], while little attention has been paid to other bacteria, such as streptococci, although they are the predominant microorganisms in the oral cavity [3] and can produce hydrogen sulfide [4].

Viridans group streptococci, which include several species, are an important part of the normal flora of the human oral and pharyngeal cavities. They are also responsible for several infections, including purulent infections [5], endocarditis [6], septicemia [7], and meningitis [8]. Recently, we reported that the βC-S lyase encoded by the *lcd* gene in *Streptococcus anginosus* catalyzes the α,β-elimination of L-cysteine to produce hydrogen sulfide [9]. In this study, we evaluated the capacities of difference of crude enzyme extracts from six species of oral streptococci to produce hydrogen sulfide, including *S. anginosus*, *Streptococcus salivarius*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus gordonii*, and *Streptococcus sobrinus*. Each βC-S lyase gene was cloned from chromosomal DNA of the streptococci and the deduced amino acid sequences were compared with that of *S. anginosus* βC-S lyase. In addition, the recombinant purified enzymes were produced and characterized.

Materials and methods

Bacterial strains, culture conditions, and genetic methods. S. anginosus FW73, *S. salivarius* HT9R, *S. mutans* XC, *S. oralis* ATCC 10557, *S. gordonii* DL1 (Challis), and *S. sobrinus* MT8246 were grown as previously described [9]. *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 10953 were grown anaerobically in GAM broth (Nissui Medical Co.). *Actinobacillus actinomycetemcomitans* NCTC 9710 was grown as previously described [10]. *Treponema denticola* ATCC 35404 was grown anaerobically in TYGVS broth [11]. *Escherichia coli* BL21 (F^- *ompT* [lon] *hsdS*$_B$($r_B^-m_B^-$) *gal dcm* λDE3) (Novagen) and M15 harboring pREP4 (*lacI* Kan^r on pREP4, F^- *recA*$^+$ *uvr*$^+$ *lon*$^+$ *lac*) (Qiagen) were grown aerobically in 2× TY broth at

☆ *Abbreviations:* GST, glutathione *S*-transferase.

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37 °C. When required, ampicillin was used to supplement the media at 100 mg/l.

Standard DNA recombinant procedures, such as DNA isolation, endonuclease restriction, and ligation, were carried out as described by Sambrook et al. [12]. Chromosomal DNA of streptococci was prepared as described previously [13].

Preparation of crude enzyme extract. Each species was grown to an optical density at 550 nm (OD_{550}) of about 0.8. The cells were harvested from 50 ml of culture and then washed with phosphate-buffered saline (0.12 M NaCl, 0.01 M Na_2HPO_4, and 5 mM KH_2PO_4 [pH 7.5]) containing 0.5 mM phenylmethylsulfonyl fluoride. A 1-ml aliquot of the cell suspension was transferred to a screwed microcentrifuge tube containing 1.0 g of 0.1–0.15-mm-diameter glass beads (Biospec Products). The cells were vortexed with the glass beads for 45 s 10 times at 1-min intervals. The supernatant was centrifuged at 18,000*g* for 30 min at 4 °C.

Cloning and sequencing of the lcd genes. Portions of the *lcd* genes from *S. salivarius*, *S. oralis*, *S. gordonii*, and *S. sobrinus* were cloned using PCR with primers designed from the *lcd* gene sequence from *S. mutans* genomic database (http://www.genome.ou.edu/smutans.html) (Table 1). A 900-bp internal portion of the *lcd* gene in *S. salivarius* was amplified using primers SMF1 and SMR5. A 700-bp internal portion of the *lcd* gene in *S. oralis* was amplified using primers SMF1 and SMR3. A 500-bp internal portion of the *lcd* gene in *S. gordonii* was amplified using primers SMF3 and SMR4. A 500-bp internal portion of the *lcd* gene in *S. sobrinus* was amplified using primers SMF3 and SMR5. Each fragment was cloned into pGEM-T Easy vector (Promega) and sequenced. Inverse PCR [14] was used to isolate DNA sequences upstream and downstream from each fragment. Briefly, genomic DNA was digested with an appropriate restriction enzyme and a dilution of the DNA sample was self-ligated. Samples from the ligation reaction were then amplified using relevant primers under standard PCR conditions. The primers were designed from the sequence of the internal portion of each *lcd* gene. The resulting amplicons were cloned into pGEM-T Easy vector and sequenced. The entire DNA sequence of the *lcd* gene in *S. mutans* was obtained from the *S. mutans* genomic DNA database.

Purification of recombinant βC-S lyase. The recombinant βC-S lyase of *S. anginosus* was obtained, as previously described [9]. The entire DNA fragment of the *lcd* gene in *S. salivarius* or *S. mutans* was amplified by PCR using primer pairs indicated in Table 1. Each amplified fragment containing the ORF for βC-S lyase was digested with *Bam*HI and *Sal*I, and then cloned in-frame with glutathione *S*-transferase (GST) into the pGEX-6P-1 vector (Amersham Biosciences). The recombinant βC-S lyases of *S. oralis*, *S. gordonii*, and *S. sobrinus* were purified using pQE60 or pQE70 (Qiagen). The DNA fragment of the *lcd* gene in *S. oralis*, *S. gordonii* or *S. sobrinus* was amplified by PCR using primer pairs listed in Table 1. The amplified fragment of the βC-S lyase ORF of *S. oralis* or *S. gordonii* was digested with *Nco*I and *Bgl*II and cloned in-frame with a 6× His tag into pQE60 vector, while that of the *S. sobrinus* βC-S lyase was digested with *Sph*I and *Bgl*II and then cloned into pQE70 vector. The resulting plasmids were pGEXSAL120 (*lcd* from *S. salivarius* [$lcd_{S.\ salivarius}$]), pMULCD110 (*lcd* from *S. mutans* [$lcd_{S.\ mutans}$]), pQEORA001 (*lcd* from *S. oralis* [$lcd_{S.\ oralis}$]), pQEGOR001 (*lcd* from *S. gordonii* [$lcd_{S.\ gordonii}$]), and pQESOB001 (*lcd* from *S. sobrinus* [$lcd_{S.\ sobrinus}$]). *E. coli* BL21 was transformed with pGEXSAL120 or pMULCD110, while *E. coli* M15 was transformed with pQEORA001, pQEGOR001 or pQESOB001. Each transformant was grown at 37 °C until an OD_{550} of 1.0 was attained. Expression was induced with 0.1 mM isopropyl-β-thiogalactopyranoside. The cells were harvested 1 h after induction and lysed by ultrasonication. The cell extract was obtained by centrifugation at 18,000*g* for 30 min at 4 °C. The *lcd* proteins from *E. coli* harboring pGEXSAL120 or pMULCD110 were purified using a MicroSpin GST Purification Module (Amersham), as previously described [9], while the *lcd* proteins from *E. coli* harboring pQEORA001, pQEGOR001 or pQESOB001 were purified using an Ni–NTA Spin Kit (Qiagen). The purification procedures followed the manufacturers' instructions. The purity of the protein was analyzed by SDS–PAGE.

Enzyme assay. The formation of hydrogen sulfide and pyruvate was measured to examine the enzymatic activity of βC-S lyase.

The formation of hydrogen sulfide was confirmed by gas chromatograph analysis, as previously described [15]. The methylene blue formation assay was used to estimate the production of hydrogen

Table 1
Oligonucleotide primers used in this study

Species	Primer	Sequence (5′ to 3′)[a]
For cloning of *lcd* genes		
	SMF1	ATGGGACGATATGATTTTACAA
	SMF3	AAGGAGATGCTGTTTTAATTAAC
	SMR2	TGAATTTCATCAGAAACTAA
	SMR3	GTTCCTGCAATATTAAAAGTTTTAGT
	SMR4	TTCATGCTGATTGTTAGCCAAC
	SMR5	AGCCAAACCAAATAAGTACCTT
For production of recombinant Lcds		
S. salivarius	SAL-LCD-forward	TCCGGATCCACACGTTATGATTTTACAACA
	SAL-LCD-reverse	GACGTCGACTTATTTTCCAAAGACAGAAA
S. mutans	MUT-LCD-forward	TCCGGATCCGGACGATATGATTTTACAAC
	MUT-LCD-reverse	GACGTCGACTTATTTTCCAAAGACACTGA
S. oralis	ORA-LCD-forward	TGGCCATGGGAAAATATAATTTTAAA
	ORA-LCD-reverse	TCTAGATCTCTTAGGCAAAACAACTCC
S. gordonii	GOR-LCD-forward	TGGCCATGGAAAGATATAATTTTGA
	GOR-LCD-reverse	TCTAGATCTTTTAGGCAAAACAGCTCCAA
S. sobrinus	SOB-LCD-forward	TGCGCATGCGTCCAGAGCTCCTTGTG
	SOB-LCD-reverse	TCTAGATCTGAAGACCTTAACCAAACGAT

[a] Nucleotides underlined in each primer sequence show the positions of the restriction endonuclease sites incorporated to facilitate cloning.

sulfide, following the method of Schmidt [16]. The reaction mixture contained the following in a final volume of 100 μl: 40 mM potassium phosphate buffer [pH 7.6], 56 mM NaCl, 2.5 mM dithioerythritol, 10 μM pyridoxal 5′-phosphate, 8 μg of the crude enzyme extract, and 0.2 mM L-cysteine. After a 10-min incubation at 37 °C, the reaction was terminated by adding 10 μl of solution I (20 mM *N*′,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl) and 10 μl of solution II (30 mM $FeCl_3$ in 1.2 M HCl). After 30 min at room temperature, the formation of methylene blue was determined spectrophotometrically at 670 nm using a molar extinction coefficient of 28.5×10^6 [16].

Pyruvate formation was detected as previously described [9,17]. The assay was carried out with 500 μl of 50 mM potassium phosphate buffer (pH 7.6) containing 5 nmol of pyridoxal 5′-phosphate, 830 ng of the purified βC-S lyase, and various amounts of L-cysteine or L-cystathionine. The kinetic parameters were computed from the Lineweaver–Burk transformation (V^{-1} versus S^{-1}) of the Michaelis–Menten equation, where V (μmol/min/mg) is the amount of pyruvate formed and S (mM) is the concentration of each substrate.

Nucleotide sequence accession numbers. The sequences reported here were submitted to the EMBL and GenBank databases through the DDBJ and assigned the following accession numbers: AB089920 ($lcd_{S.\,salivarius}$), AB089922 ($lcd_{S.\,oralis}$), AB089923 ($lcd_{S.\,gordonii}$), and AB089924 ($lcd_{S.\,sobrinus}$).

Results and discussion

Crude enzyme extracts obtained from *S. anginosus*, *S. salivarius*, *S. mutans*, *S. oralis*, *S. gordonii*, and *S. sobrinus* were tested for their capacities to form hydrogen sulfide from L-cysteine and compared with extracts from periodontopathogenic bacteria known to produce hydrogen sulfide (Fig. 1). Activity was confirmed in all the crude enzyme extracts from streptococci. Of the streptococci tested, the crude extract obtained from *S. anginosus* had the greatest capacity to produce hydrogen sulfide, and its capacity almost equaled to that of the crude extracts from *F. nucleatum* and *T. denticola*, and was much higher than those from *P. gingivalis* and *A. actinomycetemcomitans*. These results indicate that oral streptococci participate in hydrogen sulfide production in the oral cavity. From the perspective of hydrogen sulfide production, we should not ignore the contribution of *S. anginosus*.

To elucidate differences in primary structure, the *lcd* genes encoding βC-S lyase, which produces hydrogen sulfide from L-cysteine, were cloned from four streptococci (*S. salivarius*, *S. oralis*, *S. gordonii*, and *S. sobrinus*) using PCR and inverse PCR and then sequenced. The *S. mutans* homologue was obtained from the *S. mutans* genomic DNA sequence database. The deduced amino acid sequences of the genes in these five species were compared with that of *S. anginosus*. Although overall amino acid similarity was approximately 60%, no meaningful alignments producing enzymatic differences were identified in the streptococci lyases. The amino acid sequences of the streptococci Lcds were also similar to those of *T. denticola* Hly protein [17] and *E. coli* MalY

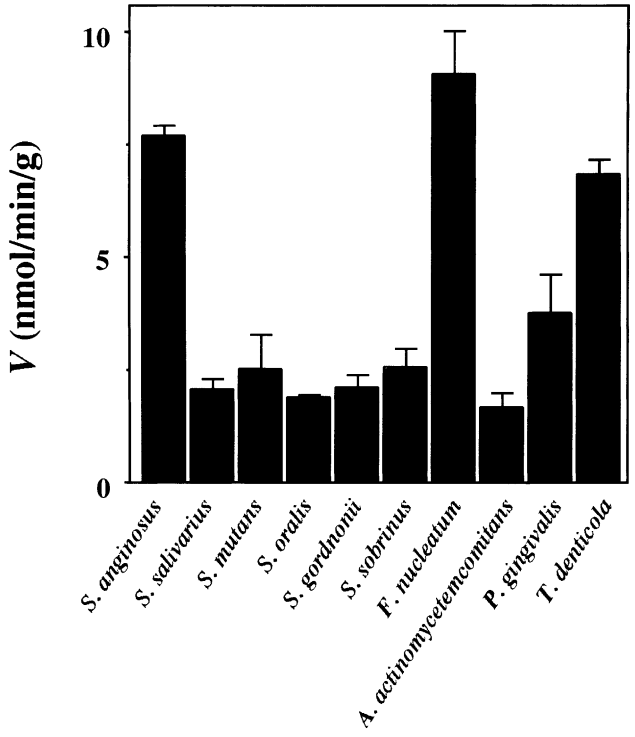

Fig. 1. Comparison of hydrogen sulfide formation by incubation of crude enzyme extracts with L-cysteine (0.2 mM). Data are means ± standard deviations of three independent experiments.

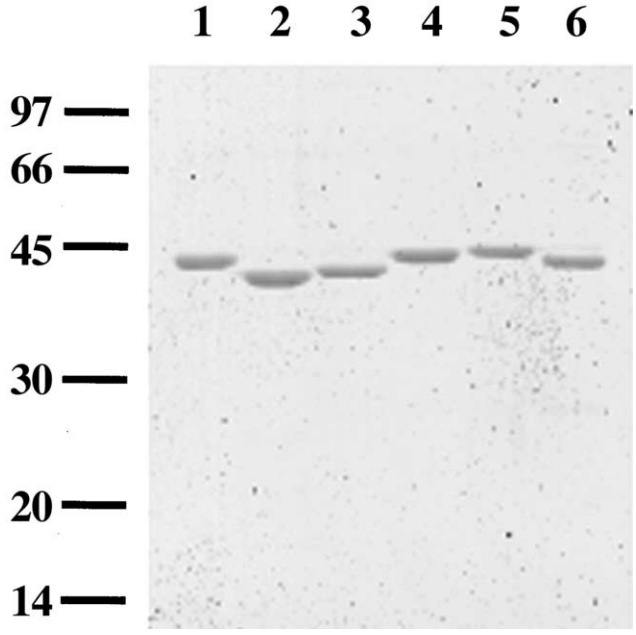

Fig. 2. PAGE analysis showing expression of the recombinant purified *lcd* products (βC-S lyases) from streptococcal species. Samples (1 μg) were subjected to SDS–PAGE and the gel was stained with a Coomassie brilliant blue. Lanes 1: the recombinant Lcd from *S. anginosus*; 2, the recombinant Lcd from *S. salivarius*; 3, the recombinant Lcd from *S. mutans*; 4, the recombinant Lcd from *S. oralis*; 5, the recombinant Lcd from *S. gordonii*; and 6, the recombinant Lcd from *S. sobrinus*. The position of molecular mass marker is given in kilodaltons.

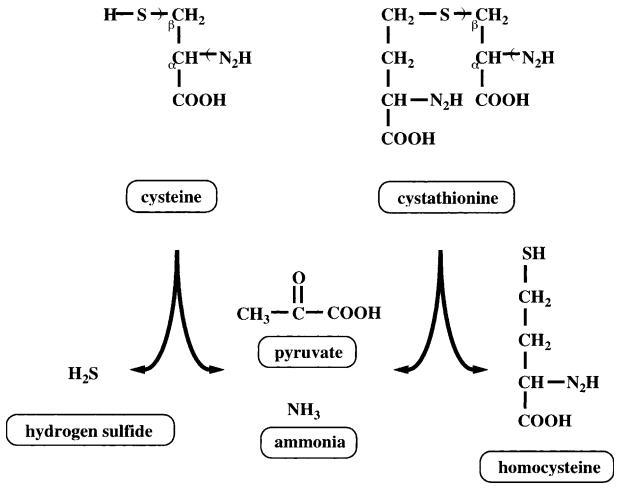

Fig. 3. Enzymatic degradation of cysteine and cystathionine by the *lcd* gene-encoded βC-S lyase. The sites of the splitting at the αC and βC atoms of the substrates are indicated. Arrows point to products released via α,β-elimination.

protein [18], which have βC-S lyase activity and form hydrogen sulfide, ammonia, and pyruvate from L-cysteine.

To evaluate the enzymatic activities of the *lcd* products, the recombinant enzyme of each species was purified using two different purification kits. SDS–PAGE analysis of each recombinant enzyme showed a single band (Fig. 2). The approximate 44-kDa molecular mass of the denatured polypeptides agreed well with the predicted molecular mass of the *lcd* proteins. Subsequently, the purified βC-S lyases were characterized. The formation of hydrogen sulfide from L-cysteine by purified βC-S lyases was confirmed by gas chromatography (data not shown). In order to evaluate the differences in the activities of purified βC-S lyases, the breakdown of L-cysteine was determined by assaying the production of pyruvate, which is a by-product of the reactions that degrade L-cysteine (Fig. 3). Like the crude enzyme samples, the purified βC-S lyase from *S. anginosus* had extremely high enzymatic activity in the presence of L-cysteine, compared with those from the other streptococci (Fig. 4A). However, the K_m of *S. anginosus* βC-S lyase for L-cysteine was not very low, compared with the other streptococcal enzymes (Table 2). By contrast, the V_{max} of *S. anginosus* βC-S lyase for L-cysteine was extremely high in comparison with the other streptococcal enzymes (Table 2). In a preliminary experiment, we

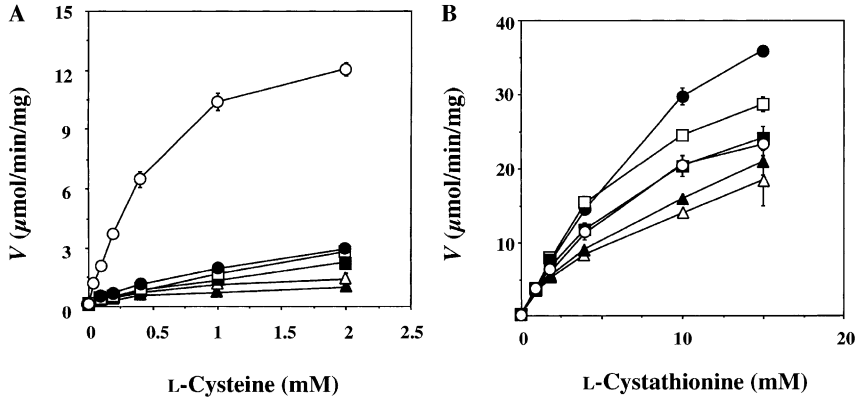

Fig. 4. Kinetics of hydrogen sulfide formation by incubation of the recombinant *lcd* products with (A) L-cysteine and (B) L-cystathionine. Open circle: Lcd from *S. anginosus*; closed circle: Lcd from *S. salivarius*; open square: Lcd from *S. mutans*; closed square: Lcd from *S. oralis*; open triangle: Lcd from *S. gordonii*; closed triangle: Lcd from *S. sobrinus*. Data are means ± standard deviations of three independent experiments.

Table 2
Kinetic properties of the *lcd* products from streptococcal species

Species	L-Cysteine		L-Cystathionine		Relative activity (%)[a]
	K_m (mM)	V_{max} (μmol/mim/mg)	K_m (mM)	V_{max} (μmol/mim/mg)	
S. anginosus	0.88	19.4	11.1	40.1	310
S. salivarius	1.16	4.33	25.5	102.4	55
S. mutans	2.46	5.53	11.8	54.4	43
S. oralis	1.08	2.43	11.2	42.6	37
S. gordonii	0.68	1.71	8.7	26.9	33
S. sobrinus	0.75	1.24	10.6	33.8	23

[a] To calculate relative activity, the velocity of degradation for 1 mM L-cysteine was divided by that for 1 mM L-cystathionine. Values are means of three determinations.

observed that higher concentrations of L-cysteine inhibited the pyruvate assay; therefore, the L-cysteine substrate concentrations were less than 2 mM. Since βC-S lyase cleaves sulfur amino acids containing βC-S linkages [9], its capacity to degrade L-cystathionine was also compared. The *S. salivarius* enzyme had the greatest activity (Fig. 4B). At low concentrations (less than 2 mM) of L-cystathionine, the βC-S lyase activities of six streptococcal species did not differ significantly (Fig. 4B). This reaction, normally catalyzed by the cystathionase (cystathionine β-lyase) encoded by *metC* [19,20], is the penultimate step in microbial methionine biosynthesis, i.e., the α,β-elimination reaction of cystathionine produces homocysteine, pyruvate, and ammonia (Fig. 3). These Lcd proteins might function as cystathionases in cells. Since the *lcd* products showed weak homology to the *metC* products of *E. coli* [19] and *Lactococcus lactis* [20], the *S. anginosus* Lcd is a cystathionase with a unique activity to degrade L-cysteine. Some investigators previously reported that the relative ability of cystathionine β-lyase in other bacteria to degrade L-cysteine was approximately 10–15% of its ability to degrade L-cystathionine [21,22]. The relative activity (310%) of *S. anginosus* Lcd also implies the uniqueness of this enzyme (Table 2). These findings showed that the difference in the capacity to produce hydrogen sulfide is due to differences in the enzymatic characteristics. However, the real reason why the *S. anginosus* Lcd was so unique is still unknown. There are no meaningful alignments specific to the *S. anginosus* Lcd. In addition, all four amino acid residues that are invariant in the comprehensive alignment of aminotransferases belonging to the α family of pyridoxal 5′-phosphate-dependent enzymes [23] are conserved in all the *lcd* proteins (data not shown). The secondary or tertiary structure of *S. anginosus* Lcd might differ from those of the others' Lcds.

S. anginosus Lcd is very interesting, not only from the viewpoint of enzymology, but also from the perspective of the virulence of the organism. *S. anginosus* is frequently isolated from certain infectious sites and is thought to be associated with abscess formation [5,24,25]. However, the relationship between abscess formation and the role of *S. anginosus* is not known. The βC-S lyase in *S. anginosus* might play an important role in abscess formation, since hydrogen sulfide causes the modification and release of hemoglobin in erythrocytes [9], and the concentration of free cysteine in human serum is very high (0.26 mM), compared with other sulfur compounds, such as L-methionine (0.026 mM) and L-homocysteine (0.013 mM) [26].

In conclusion, the six species of oral streptococci that we tested have the capacity to form hydrogen sulfide in the oral cavity. Of these, *S. anginosus* is the most important bacteria for hydrogen sulfide production. The capacity of *S. anginosus* to form hydrogen sulfide is due to the unique characteristics of its βC-S lyase. Further studies are necessary to clarify the unique characteristics of the *S. anginosus* βC-S lyase including its relationship to the virulence of the organism.

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