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Molecular and enzymatic characterization of βC-S lyase in *Streptococcus constellatus*

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Background/aims: *Streptococcus anginosus* and *Streptococcus constellatus* are frequently isolated from dental abscesses and other suppurative lesions. We previously reported that βC-S lyase from a strain of *S. anginosus* produced significantly more hydrogen sulfide than βC-S lyases from other streptococci. The purpose of this study was to establish the molecular and enzymatic features of the βC-S lyase in *S. constellatus* and to elucidate whether this unique capacity is common to many strains of *S. constellatus* and *S. anginosus*.

Methods: The capacity of crude extract to produce hydrogen sulfide was evaluated among 16 strains of *S. constellatus*, *S. anginosus*, and *Streptococcus gordonii.* The *lcd* gene encoding βC-S lyase was cloned from the genomic DNA of each strain to compare the deduced amino acid sequences. The recombinant βC-S lyases of three representative strains were purified and characterized.

Results: Incubation of crude extracts from all strains of *S. constellatus* and *S. anginosus* with L-cysteine resulted in the production of a large amount of hydrogen sulfide. The primary sequence of βC-S lyase was very similar among strains of *S. constellatus* and *S. anginosus*. The kinetic properties of the βC-S lyases purified from *S. constellatus* resembled those for βC-S lyases purified from *S. anginosus*. In contrast, the βC-S lyases of *S. constellatus* and *S. gordonii* differed in terms of their hydrogen sulfide production, with the former producing much more.

Conclusion: A high level of hydrogen sulfide production, which appears to be a common feature in both *S. constellatus* and *S. anginosus*, may be associated with their abscess formation.

Key words: βC-S lyase; hydrogen sulfide; pyridoxal-5′-phosphate-dependent enzyme; *Streptococcus anginosus* group; *Streptococcus constellatus*

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The anginosus group of streptococci, which is also referred to as the milleri group, consists of several related species of *Streptococcus anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (14, 33). The members of this group are generally regarded as commensal microflora of the body and are found at various sites including the mouth, genitourinary tract, and gastrointestinal tract (25). They are also frequently encountered in suppurative infections at a range of clinical sites, such as liver and brain abscesses, and along with *Streptococcus oralis*, they are involved in infective endocarditis (11, 13, 21, 32, 34). Thus, these species are considered to share a remarkable propensity to cause abscesses (6, 13, 34). Despite increasing awareness of the clinical significance of the anginosus group, little is known about the pathogenesis, virulence factors, or protective antigens.

The βC-S lyases are pyridoxal-5′-phosphate (PLP)-dependent enzymes that catalyze the α,β-elimination of sulfur amino acids containing αC-N and βC-S linkages, such as L-cysteine, L-cystathionine, L-cystine, *S*-(2-aminoethyl)-L-cysteine, and *S*-methyl-L-cysteine, to the sulfur-containing molecules, pyruvate and ammonia (12) (Fig. 1). βC-S lyase is encoded by the *lcd* gene. Cystathionine γ-synthase, which is encoded by the *cgs* gene, is cotranscribed with *lcd*, and it functions in the biosynthesis of methionine in *S. anginosus* (36, 38). In this pathway,

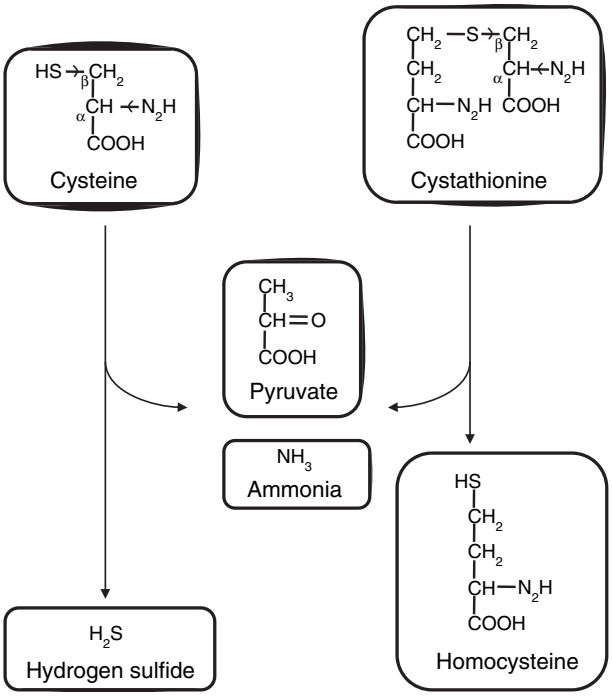

Fig. 1. Enzymatic degradation of cysteine and cystathionine by the *lcd* gene-encoded βC-S lyase. The sites of the splitting at the αC and βC atoms of the substrates are indicated. Arrows point to products released via α,β-elimination.

the βC-S lyase cleaves L-cystathionine formed from L-cysteine by Cgs, to produce homocysteine, which is subsequently methylated to form methionine. Interestingly, the βC-S lyase from a single laboratory strain of *S. anginosus* (strain FW73) was shown to produce an extraordinary amount of hydrogen sulfide from L-cysteine, compared to βC-S lyases from various oral streptococci, such as *Streptococcus gordonii*, *S. oralis*, *Streptococcus mutans*, *Streptococcus sobrinus*, and *Streptococcus salivarius* (37). Because hydrogen sulfide is highly toxic to mammalian cells (4) and induces the modification and release of hemoglobin from erythrocytes (16, 36), abscess formation by members of the anginosus group of streptococci may be associated with elevated hydrogen sulfide production. However, it not known whether this unique capacity is common to the other strains of this species or to the two remaining species of the anginosus group *S. constellatus* and *S. intermedius*.

In this study, crude extracts from reference and clinical strains of *S. constellatus* and *S. anginosus* were evaluated for their capacity to degrade L-cysteine to hydrogen sulfide. The *lcd* genes encoding βC-S lyase were cloned from all of the anginosus group strains tested, and then sequenced. Moreover, recombinant *S. constellatus* and *S. anginosus* proteins were purified and characterized.

Materials and methods

Bacterial strains, culture conditions, and genetic methods

Streptococcus constellatus and *S. anginosus* isolates indicated by the prefix IMU were collected from clinical specimens submitted for culture to the Division of the Central Clinical Laboratory, Iwate Medical University Hospital, Morioka, Japan (Table 1). All of the strains were isolated from distinct specimens. The assignment of each strain to either *S. constellatus* or *S. anginosus* was confirmed by polymerase chain reaction (PCR), as previously described (30). The type strains of the anginosus group of streptococci (i.e. *S. constellatus* ATCC 27823 and *S. anginosus* ATCC 33397) were also included in this study. Clinical isolates of *S. gordonii*, which were obtained from three healthy volunteers, were identified by sequencing the *sodA* gene (15). The source of *S. gordonii* Challis has been described elsewhere (38). The streptococci were grown anaerobically in brain–heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C. The *Escherichia coli* strains DH5α (Invitrogen, Carlsbad, CA) and BL21 (Promega, Madison, WI), which were used for DNA manipulation and protein purification, respectively, were grown aerobically in 2 × tryptone–yeast extract broth at 37°C. When required, kanamycin or ampicillin was added to the media at 100 μg/ml.

Preparation of crude enzyme extracts

Crude enzyme extracts were obtained as previously described (37). Briefly, each strain was grown to an optical density at 600 nm (OD_{600}) of about 0.8. The cells were then harvested from 200 ml culture and washed with phosphate-buffered saline (0.12 M NaCl, 0.01 M Na_2HPO_4, 5 mM KH_2PO_4, pH 7.5) three times. A 500-μl aliquot of the cell suspension

Table 1. Streptococcal strains used in this study

Strain	Isolation source/description	Accession no. assigned	
		16S rRNA	*lcd*
S. constellatus ATCC 27823	Type strain	AB355605	AB327000
S. constellatus IMU104	Ascites fluid	AB355606	AB327001
S. constellatus IMU106	Abscess, abdomen	AB355607	AB327002
S. constellatus IMU108	Abscess, mouth	AB355619	AB327003
S. constellatus IMU116	Pleural fluid	AB355608	AB327005
S. constellatus IMU120	Abscess, throat	AB355618	AB327006
S. anginosus ATCC 33397	Type strain	AB355609	AB326994
S. anginosus IMU102	Abscess, skin	AB355610	AB326995
S. anginosus IMU103	Abscess, abdomen	AB355611	AB326996
S. anginosus IMU107	Abscess, tonsil	AB355612	AB326997
S. anginosus IMU112	Abscess, peritoneum	AB355613	AB326998
S. anginosus IMU114	Abscess, skin	AB355614	AB326999
S. gordonii Challis	Laboratory strain	AB355601	AB089923
S. gordonii NUK38	Supragingival dental plaque	AB355602	AB327007
S. gordonii NJ122	Supragingival dental plaque	AB355603	AB327008
S. gordonii NY126	Supragingival dental plaque	AB355604	AB327009

The clinical strains of the anginosus group of streptococci identified using Streptogram (Wako) were precisely assigned to *S. anginosus* or *S. constellatus* by the method of Takao et al. (30). The strains of *S. gordonii* that were isolated on Todd–Hewitt agar plates were identified by sequencing *sodA* (15). The identification was supported by sequencing of the 16S rRNA gene.

was transferred to a screw-cap microcentrifuge tube containing 0.5 *g* glass beads with diameters between 0.1 and 0.15 mm. After vortexing the cells with the glass beads 10 times for 30 s each time at 1-min intervals, the supernatant was centrifuged. The concentration of proteins in the crude extracts was determined using a protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The samples were stored at −20°C after adding an equal volume of 80% glycerol.

Cloning and sequencing of the *lcd* and 16S ribosomal RNA (rRNA) genes

PCR fragments (*c*. 1.3-kb) containing the *lcd* gene of the *S. constellatus* or *S. anginosus* strains were cloned using primers (Table 2) designed based on the flanking regions of the *S. intermedius* UOEH301 *lcd* gene (Y. Yoshida & S. Ito, unpublished data; Accession no. AB271060). The corresponding fragments from three clinical strains of *S. gordonii* were amplified using primers (Table 2) designed on the basis of the *S. gordonii* Challis genomic database (http://www.tigr.org/). The partial sequence (1.4 kb) of the 16S rRNA gene was PCR amplified as previously described (29). The primers used are shown in Table 2. Each amplified fragment was cloned into pCR-Blunt II-TOPO (Invitrogen), and then sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were assembled and analyzed using VECTOR NTI software (Invitrogen).

Reverse transcription (RT)-PCR analysis

Total RNA was isolated from streptococcal cells using FastPrep Blue tubes (MP Biomedicals, Solon, OH), as previously described (38). Contaminating DNA was eliminated by digestion with RNase-free DNase (Takara Bio, Otsu, Japan). RNA (10 ng) was reverse transcribed into single-stranded complementary DNA using PrimeScript Reverse Transcriptase (Takara Bio Inc.), according to the manufacturer's instructions. The gene-specific primers used in RT-PCR are listed in Table 2. Each reverse primer for RT-PCR analysis was also used to synthesize complementary DNA from specific mRNA in total RNA. Reaction mixtures that were used as negative controls contained no reverse transcriptase to evaluate the presence of contaminated genomic DNA in the samples.

Purification of recombinant βC-S lyases

Recombinant Lcd from *S. constellatus* ATCC 27893, *S. constellatus* IMU106, and *S. anginosus* ATCC 33397 were purified using the expression vector pGEX-6P-1 (GE Healthcare, Buckinghamshire, UK) as previously described (36). Briefly, each *lcd* gene was amplified by PCR with primers containing *Bam*HI, *Sal*I, or *Xho*I sites at their 5′ ends (Table 2). Each amplicon was digested with the appropriate restriction enzymes, and then inserted in-frame with the glutathione *S*-transferase gene into the same sites of pGEX-6P-1. The recombinant *E. coli* clones were grown in 2× tryptone–yeast extract broth containing ampicillin to an OD_{600} of about 0.8, and isopropyl-β-thiogalactopyranoside was added at a final concentration of 1 mM. The cells were harvested 2 h after induction, suspended in phosphate-buffered saline, and lyzed by ultrasonication. Cell lysates were collected after centrifugation at 15,000 *g* for 30 min at 4°C and incubated with glutathione Sepharose 4B beads (GE Healthcare). After extensive washing, the beads were treated with PreScission protease (GE Healthcare) and Lcd was recovered. The protein concentration was determined using the Bio-Rad protein assay reagent. The purity of the samples was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Visualization of enzymatic activity

L-Cysteine desulfhydrase activity was visualized in non-denaturing polyacrylamide gels as previously described (5).

Table 2. Oligonucleotide primers used in this study

Designation	Sequence (5′ to 3′)	Target specificity
For cloning of *lcd*		
Lcdgene-Forward1	ACAGATTTAACATTTGCTTTGGAGGT	*S. anginosus* strains ATCC 33397, IMU102, IMU103, IMU107, IMU112 and IMU114
Lcdgene-Forward2	CTAGCGAAGATATCAAGATGCTATGG	*S. constellatus* strains ATCC 27823, IMU104, IMU106, IMU108, IMU116 and IMU120
Lcdgene-Forward3	GGGAGGAGTGGAGAGTTTGAT	*S. gordonii* strains NUK38, NY122 and NY126
Lcdgene-Reverse1	CTCCACTTAAGAATGATTGT	*S. anginosus* strains ATCC 33397, IMU102, IMU103, IMU107, IMU112 and IMU114 and *S. constellatus* strains ATCC 27823, IMU106, IMU108, IMU116 and IMU120
Lcdgene-Reverse2	CATACTCCACTTAAGAATGATTGTCAA	*S. constellatus* IMU104
Lcdgene-Reverse3	GGTTTCTTGCATAGGAAGCTG	*S. gordonii* strains NUK38, NY122 and NY126
For sequencing of 16S rRNA gene		
16S-Foward	GGAATTCAGAGTTTGATCMTGGYTCAG	All strains used in this study
16S-Reverse	CGGGATCCAAGGAGGTGATCCADCCVCA	All strains used in this study
For reverse transcription–polymerase chain reaction		
RT-Sc-Forward	AGCAAATACAATTTTCAAACAG	*S. constellatus* ATCC 27823
RT-Sc-Reverse	ATCCAACACCGCTTGGA	*S. constellatus* ATCC 27823
RT-Sa-Forward	CCGAATCGCTTATCTCATC	*S. anginosus* ATCC 33397
RT-Sa-Reverse	GCTTGGAGCAACTCATCA	*S. anginosus* ATCC 33397
RT-Sg-Forward	GAGCTTGATAGAGCGAGCA	*S. gordonii* Challis
RT-Sg-Reverse	ATACCTATGCTAGTGAGGAGTTGAT	*S. gordonii* Challis
For production of the recombinant Lcd		
Lcdprotein-Forward1	TCCGGATCCAGCAAATACAATTTTCAAACAG	*S. anginosus* ATCC 33397 and *S. constellatus* strains ATCC 27823 and IMU106
Lcdprotein-Reverse1	TTAGTCGACTTATTGTGGCAAACAATGC	*S. anginosus* ATCC 33397
Lcdprotein-Reverse2	TTACTCGAGTTATTTGGGCAAGCAACACAC	*S. constellatus* ATCC 27823
Lcdprotein-Reverse3	TTACTCGAGTTATTTGGGCAAACAACGC	*S. constellatus* IMU106

Nucleotides underlined in each primer show the positions of the restriction endonuclease sites incorporated to facilitate cloning.
M, nucleotides A and C; Y, nucleotides C and T; D, nucleotides A, G and T; V, nucleotides A, C and G.

The samples were electrophoresed at 10 mA per gel at 4°C for 3 h on 12.5% resolving (pH 8.8) and 3% stacking (pH 6.5) polyacrylamide gels. After electrophoresis, the gel was incubated in visualizing solution [100 mM triethanolamine–HCl, 10 μM PLP, 0.5 mM bismuth trichloride, 10 mM ethylenediamineteraacetic acid (EDTA) and 5.0 mM L-cysteine, pH 7.6] at 37°C to produce a black band at the position of the enzyme.

Enzyme assay

The enzymatic activity of the βC-S lyases was examined by measuring the formation of hydrogen sulfide or pyruvate. A methylene blue formation assay was performed to estimate the production of hydrogen sulfide, following the method of Schmidt (26). Briefly, the reaction mixture contained the following in a final volume of 200 μl: 40 mM potassium phosphate buffer (pH 7.6), 2.5 mM dithioerythritol, 10 μM PLP, 2.0 mM L-cysteine, and 53.6 μg crude enzyme extract. After a 10-min incubation at 37°C, the reaction was terminated by adding 20 μl solution I (20 mM *N*′,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl) and 20 μl solution II (30 mM $FeCl_3$ in 1.2 M HCl). After incubation for 30 min at room temperature, methylene blue formation was determined spectrophotometrically at 670 nm using the molar extinction coefficient of 28.5×10^6 (/M/cm).

Pyruvate formation was detected as previously described (28). The assay was carried out in a reaction mixture (100 μl) of 50 mM potassium phosphate buffer (pH 7.6) containing 1 nmol PLP, 170 or 40 ng purified enzyme and various amounts of L-cysteine or L-cystathionine. After a 2-min incubation at 37°C, the reaction was terminated by adding 50 μl of 4.5% trichloroacetic acid. The reaction mixture was centrifuged, and 100 μl of the supernatant was added to 300 μl of 0.67 M sodium acetate (pH 5.2) containing 0.017% 3-methyl-2-benzothiazolinone hydrazone. After incubation for 30 min at 50°C, the amount of pyruvate was determined at OD_{335}. 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 formation of pyruvate and S (mM) is the concentration of each substrate.

The effects of various inhibitors were determined by preincubating the purified enzyme fraction with each inhibitor at the indicated concentration in assay buffer for 10 min at room temperature. Enzymatic activity was determined in the presence of 1 mM L-cysteine or L-cystathionine, as described above.

Statistical analysis

The data obtained were analyzed by Student's *t*-test. A *P*-value of <0.01 was considered significant.

Nucleotide sequence accession number

The sequences of the *lcd* and16S rRNA genes reported here were submitted to the EMBL and GenBank databases through the DDBJ. The accession numbers are listed in Table 1.

Results

Hydrogen sulfide production capacity of the crude enzyme extract

One reference strain and five clinical strains each for *S. constellatus* and *S. anginosus* were tested for their capacity to produce hydrogen sulfide from L-cysteine. The capacity of each of the crude extracts was significantly higher (4.8–29.6 times) than that of *S. gordonii* Challis (Fig. 2), which is known to have a low capacity for hydrogen sulfide production, similar to the other oral streptococci, including *S. salivarius*, *S. mutans*, *S. oralis*, and *S. sobrinus* (37). Like *S. gordonii* Challis, incubation of crude extracts of the clinical strains of *S. gordonii* with L-cysteine resulted in only a little hydrogen sulfide production. The results suggested that abundant hydrogen sulfide production from L-cysteine is a common property of *S. constellatus* and *S. anginosus*. Cysteine desulfhydrase activity in the crude extracts of *S. constellatus* ATCC 27823, *S. constellatus* IMU106, and *S. anginosus* ATCC 33397 was assayed by *in situ* staining. Hydrogen sulfide reacts with bismuth to produce an insoluble product, which forms brown–black bands on nondenaturing polyacrylamide gels (5). Each crude extract contained a single band associated with hydrogen sulfide production from L-cysteine (Fig. 3B). The findings also suggested that no other enzymes in the cells interfered with the assay.

Cloning and molecular analysis of the *lcd* gene

The gene responsible for L-cysteine desulfhydration was amplified by PCR using genomic DNA obtained from all strains of *S. constellatus*, *S. anginosus*, and *S. gordonii* and then sequenced. All *lcd* homologues in the sequenced fragment were 1164 bp long. A possible Shine–Dalgarno sequence (27) was identified just upstream of the potential start codon of each gene. It is noteworthy that the intact *lcd* gene was also conserved in all the tested strains of *S. gordonii*, crude enzymes of which had a low capacity to produce hydrogen sulfide from L-cysteine (Fig. 2). RT-PCR analysis showed expression of the *lcd* gene in *S. gordonii*, suggesting that the low production of hydrogen sulfide by *S. gordonii* was not attributed to transcriptional interference on the *lcd* gene (Fig. 4).

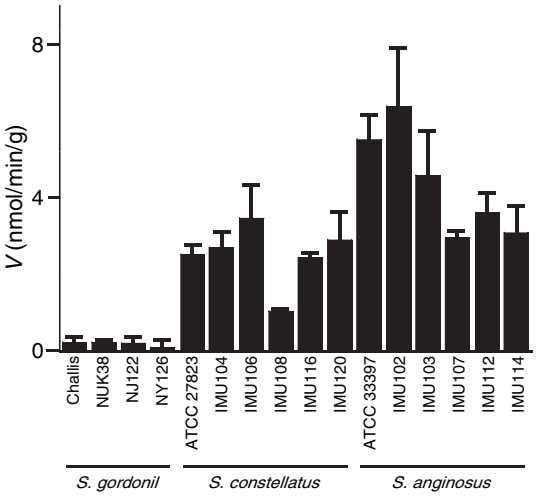

Fig. 2. Comparison of hydrogen sulfide formation by incubating crude enzyme extracts with L-cysteine (1 mM). Data are given as the means ± standard deviations of three independent experiments.

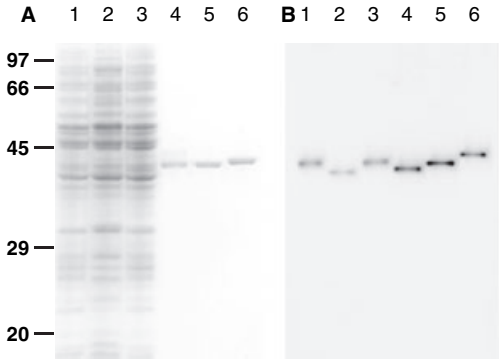

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) analyses showing the expression of the *lcd* genes from *S. constellatus* ATCC 27823, *S. constellatus* IMU106, and *S. anginosus* ATCC 33397. (A) Samples were subjected to sodium dodceyl sulfate (SDS)–PAGE, and the gel was subsequently stained with Coomassie brilliant blue. (B) Samples were subjected to non-denaturing PAGE, and the enzyme activity was visualized. The resolving gel contained 12.5% acrylamide. Lane 1, cell lysate of *S. constellatus* ATCC 27823; lane 2, cell lysate of *S. constellatus* IMU106; lane 3, cell lysate of *S. anginosus* ATCC 33397; lane 4, recombinant purified βC-S lyase of *S. constellatus* ATCC 27823; lane 5, recombinant purified βC-S lyase of *S. constellatus* IMU106; and lane 6, recombinant purified βC-S lyase of *S. anginosus* ATCC 33397. The positions of the molecular mass markers are indicated (in kDa).

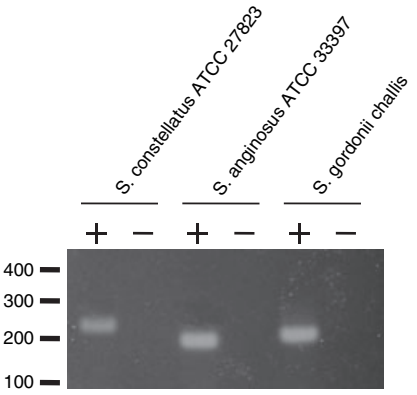

Fig. 4. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the *lcd* gene expression in exponentially growing cultures of streptococcal strains. Total RNA was prepared from mid-log-phase cultures of *S. constellatus* ATCC 27823, *S. anginosus* ATCC 33397, and *S. gordonii* Challis. Lanes marked with + are standard RT-PCR amplifications. Lanes marked with − are negative controls that contained no cDNA. The positions of DNA size standards (in bp) are indicated on the left.

A phylogenetic tree based on *lcd*, which was constructed with the corresponding published sequences of several streptococci, was compared with a phylogenetic tree based on the 16S rRNA gene sequences (Fig. 5). The trees were constructed using the neighbor-joining method. The rate of genetic divergence of the *lcd* sequence differed slightly from that of the 16S rRNA sequence. Compared with the tree of the *lcd* genes, the analysis of the 16S rRNA genes showed a 2- to 11-fold increase in the length of branches between strains of *S. constellatus* and *S. anginosus*, suggesting that the *lcd* gene was more highly conserved in *S. constellatus* and *S. anginosus* strains than the 16S rRNA gene.

Greater than 89.9% amino acid sequence identity was noted among the respective species, *S. constellatus*, *S. anginosus*, or *S. gordonii*, and the consensus sequence of *S. constellatus* was 92.0% identical to that of *S. anginosus.* These results show that the primary sequence encoded by *lcd* is also highly conserved in the seemingly unrelated strains of *S. constellatus* and *S. anginosus*. An interrogation of the database, using the consensus amino acid sequence of *S. constellatus* Lcd, revealed significant homology to previously reported βC-S lyases, including the YtjE protein of *Lactococcus lactis* (59%) (18), the PatB protein of *Bacillus subtilis* (38%) (3), the PatC protein of *Lactobacillus delbrueckii* (34%) (2), and the MalY protein of *E. coli* (29%). Amino acid sequence alignment showed that the Lys-233 residue by which the cofactor PLP is covalently attached to MalY, as well as many residues that had been reported to stabilize the cofactor, were conserved in the Lcd of all the tested strains of *S. constellatus* (7). Of three residues (Tyr-121, Asp-201, and Ser-36), which are essential for the MalY β-lyase activity, the first two residues were also conserved in Lcd from the *S. constellatus* strains, while the last one was substituted by Ala in those strains. PLP-dependent enzymes have been classified into α, β, and γ families based on sequence alignments and the construction of protein profiles (1, 19, 20). The alignment of amino acid sequences of the *S. constellatus* Lcd with the other PLP-dependent enzymes indicated that the product belonged to the α family of PLP-dependent enzymes (36).

Purification and characterization of the βC-S lyase

To obtain purified proteins for enzymatic analysis, the entire *lcd* genes from *S. constellatus* ATCC 27823, *S. constellatus* IMU106, and *S. anginosus* ATCC 33397 were amplified by PCR and cloned in-frame with glutathione *S*-transferase into pGEX-6P-1. The resulting plasmids were then used to transform competent *E. coli* BL21 cells. Each purified protein was obtained by cleaving the glutathione *S*-transferase–fusion protein bound with glutathione–Sepharose 4B medium. SDS–PAGE analysis (Fig. 3A) indicated that the purity of the recombinant Lcd agreed well with the predicted molecular mass (44 kDa). By non-denaturing PAGE, the purified recombinant Lcd proteins were shown to be associated with the production of hydrogen sulfide from L-cysteine (Fig. 3B). To evaluate the βC-S lyase activity of the purified Lcd proteins, the breakdown of L-cysteine and L-cystathionine was determined by assaying the production of pyruvate, which is a by-product of the reactions that degrade these substrates. The kinetics for the decomposition of these substrates by the Lcd proteins is shown in Fig. 6. The capacities of the purified βC-S lyases from the three strains of the anginosus group to degrade L-cysteine were comparable and significantly higher than that of *S. gordonii*. When incubated with L-cystathionine, βC-S lyase catalyzes α,β-elimination of L-cystathionine to homocysteine, pyruvate, and ammonia (36). The capacity of the anginosus group βC-S lyases to degrade L-cystathionine was also high, although it was not as great as that of the βC-S lyase from *S. gordonii*. The K_m and V_{max} values of the βC-S lyases, which were calculated from Lineweaver–Burk plots, are summarized with those from *S. gordonii* in Table 3. The values for both L-cysteine and L-cystathionine were comparable among the three βC-S lyases from the strains of the anginosus group of streptococci. These results suggest that the relative activity of Lcd from the anginosus group favors L-cysteine over L-cystathionine as a substrate.

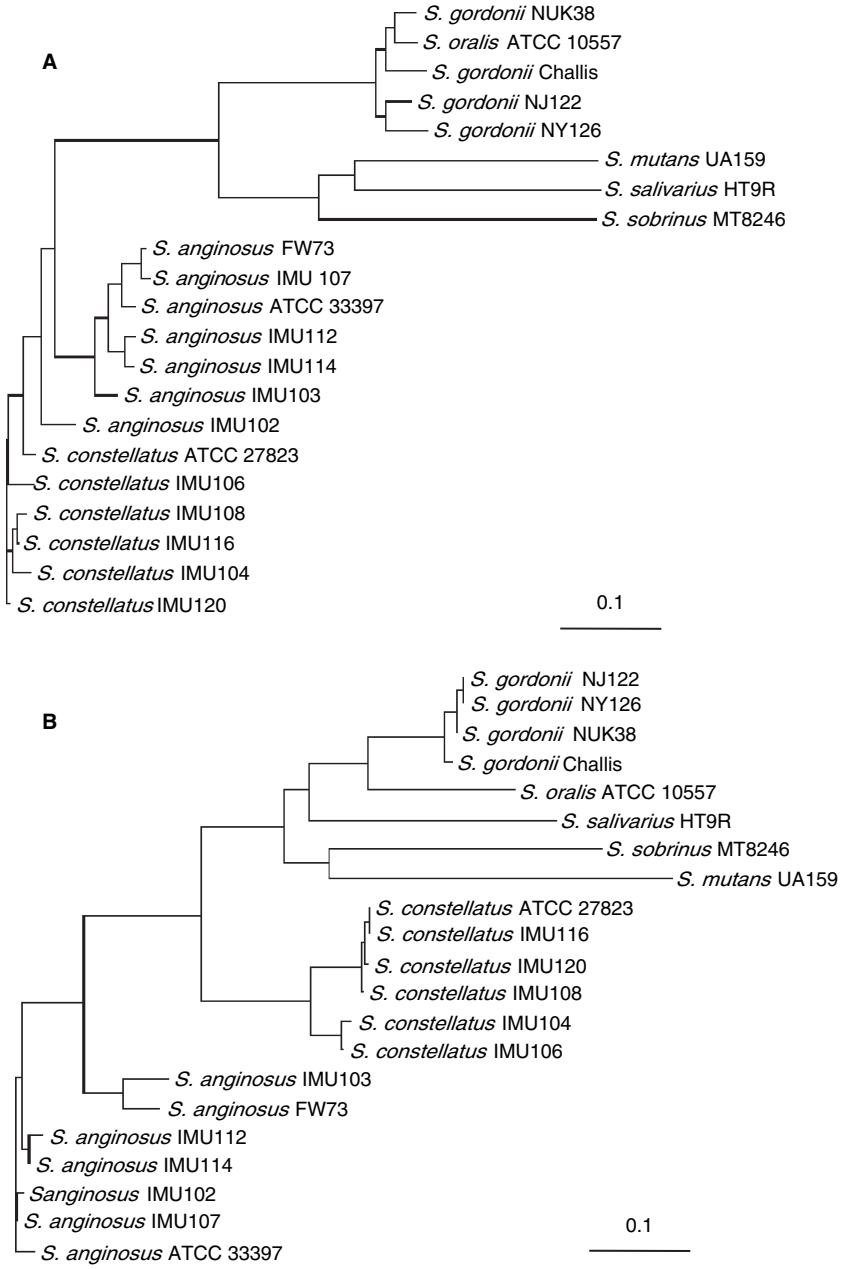

Fig. 5. Phylogenetic trees based on DNA sequences of (A) the *lcd* gene and (B) 16S rRNA gene. The trees were constructed by the neighbor-joining method, using the computer program CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and TREEVIEW X (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/). The scale indicates the percentage of nucleotide difference (i.e. percent divergence). The sequence data for the *lcd* gene from *S. anginosus* FW73, *S. oralis* ATCC 10557, *S. salivarius* HT9R, *S. mutans* UA159, and *S. sobrinus* MT8246 and for the 16S rRNA gene from *S. mutans* UA159 were taken from the GenBank database.

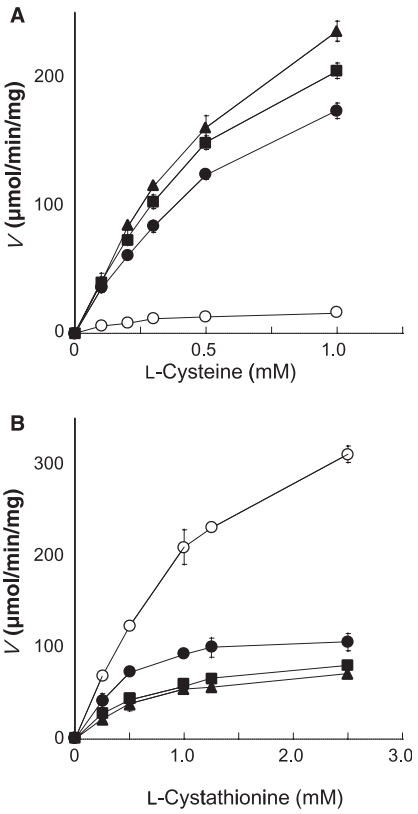

Fig. 6. Kinetics of pyruvate formation by incubating the recombinant *lcd* product from *S. constellatus* ATCC 27823 (closed square), *S. constellatus* IMU106 (closed triangle), *S. anginosus* ATCC 33397 (closed circle), or *S. gordonii* Challis (open circle) with L-cysteine (A) or L-cystathionine (B). The data are given as the means ± standard deviations of three independent experiments.

The effects of selected inhibitors on the activity of the βC-S lyase from *S. constellatus* ATCC 27823 are shown in Table 4. Both L-cysteine and L-cystathionine were used to compare the inhibitory effect on the different substrates. The enzymatic activity of Lcd was strongly inhibited by carbonyl reagents such as hydroxylamine and 3-methyl-2-benzothiazolinone hydrazone, which are known inhibitors of PLP-dependent enzymes. Another carbonyl reagent, DL-penicillamine, also had an inhibitory effect on the activity of the enzyme. DL-Cycloserine inhibited the action of *S. constellatus* Lcd, while DL-propargylglycine, the chelating reagent EDTA, and NaCl had no inhibitory effect. The sulfhydryl-reactive agent iodoacetic acid strongly inhibited the activity of Lcd, suggesting that a thiol group is essential for the activity. A difference in the substrate, either L-cysteine or L-cystathionine, did not greatly influence the effect of the inhibitors. Similar results were obtained for the βC-S lyase of *S. anginosus* ATCC 33397 (data not shown).

Discussion

The βC-S lyase from a single strain of *S. anginosus* was previously shown to have a high capacity for degrading L-cysteine and L-cystathionine to hydrogen sulfide and homocysteine, respectively (37). Our data suggest that this enzymatic property appears to be common to the strains of *S. constellatus* and *S. anginosus.* Indeed, the nucleotide sequence of *lcd* and the amino acid sequence of *lcd*-encoded βC-S lyase, which is the only enzyme associated with the production of hydro-

Table 3. Kinetic properties of the *lcd* products from streptococcal species

	L-Cysteine		L-Cystathionine	
	K_m (mM)	V_{max} (μmol/min/mg)	K_m(mM)	V_{max} (μmol/min/mg)
S. constellatus ATCC 27823	0.67	330	0.71	104
S. constellatus IMU106	0.99	492	0.87	99
S. anginosus ATCC 33397	0.99	354	0.41	130
S. gordonii Challis	0.23	19.1	0.48	526

Values are given as the means of three determinations.

Table 4. Effects of inhibitors on βC-S lyase activity of the Lcd proteins from *S. constellatus* ATCC 27823

		Relative activity of degradation for (%)[1]	
Inhibitor	Inhibitor concn	L-Cysteine	L-Cystathionine
Hydroxylamine	1 mM	7.7 ± 0.4	12.7 ± 0.3
	10 mM	0.0 ± 0.3	2.0 ± 0.6
3-Methyl-2-benzothiazolinone hydrazone	1 mM	75.4 ± 2.0	85.0 ± 2.0
	10 mM	12.6 ± 0.8	12.8 ± 0.6
DL-Penicillamine	1 mM	98.4 ± 1.0	93.5 ± 1.2
	10 mM	71.1 ± 0.8	72.5 ± 1.1
DL-Propargylglycine	1 mM	106.7 ± 3.6	101.8 ± 2.2
	10 mM	108.0 ± 0.1	96.0 ± 0.4
DL-Cycloserine	1 mM	75.9 ± 1.5	77.7 ± 2.3
	10 mM	40.5 ± 1.2	38.7 ± 0.5
Iodoacetic acid	1 mM	83.3 ± 1.0	83.8 ± 1.5
	10 mM	9.1 ± 0.3	12.2 ± 1.1
EDTA	1 mM	107.7 ± 1.4	106.8 ± 2.9
	10 mM	110.0 ± 1.9	104.8 ± 1.0
NaCl	1 mM	97.2 ± 1.0	99.1 ± 2.8
	10 mM	103.1 ± 1.9	103.2 ± 1.5

[1]100% activity is the enzyme activity toward L-cysteine or L-cystathionine at the concentration of 1 mM with no inhibitor added.
Data are given as the means ± standard errors of three independent experiments.

gen sulfide from L-cysteine in strains of both species, were highly conserved in those strains. By contrast, the βC-S lyases from the *S. gordonii* strains did not resemble those from the *S. constellatus* or *S. anginosus* strains; instead, they showed similarity with those from common oral streptococci, such as *S. oralis*, *S. salivarius*, *S. mutans*, and *S. sobrinus*. However, the low production of hydrogen sulfide by *S. gordonii* was not attributed to disruption of transcript because the gene was expressed in *S. gordonii* as well as in *S. constellatus* and *S. anginosus* (Fig. 4). The findings are not surprising because homocysteine that is synthesized from cystathionine by βC-S lyase is an intermediate indispensable for the methionine biosynthetic pathway.

The migration rate of the purified Lcd product from *S. anginosus* ATCC 33397 in SDS–PAGE was slightly different from the rates of those products from *S. constellatus* strains (Fig. 3A). This may be a result of differences in the primary structure of the Lcd protein. In native-PAGE (Fig. 3B), the migration rate of purified recombinant Lcd did not perfectly agree with that of L-cysteine desulfhydrase in the respective crude enzyme extract. This difference may be attributed to the effect of five amino acid residues (Gly-Pro-Leu-Gly-Ser) attached to the N-terminal of each protein, which are added for the production of recombinant proteins or by posttranscriptional modification of the Lcd product.

The K_m values (0.67–0.99 mM) of Lcd from *S. constellatus* ATCC 27823, *S. constellatus* IMU106, and *S. anginosus* ATCC 33397 for L-cysteine were all comparable to those from the other oral streptococci, including *S. salivarius* (1.16 mM), *S. oralis* (1.08 mM), and *S. sobrinus* (0.75 mM), although the value from the *S. mutans* Lcd was exceptionally high (2.46 mM) (37). In contrast, the K_m value of Lcd from *S. gordonii* Challis was much lower (0.23 mM), indicating that the enzyme from *S. gordonii* exhibited a high affinity to L-cysteine. Nevertheless, the V_{max} of *S. gordonii* Lcd for the same substrate was much lower compared with those from the anginosus group strains described above (<5.7%). On the other hand, the V_{max} of *S. gordonii* Lcd for L-cystathionine was at least four times higher. Since pyruvate and ammonia, which are common by-products in the degradation reaction of L-cysteine and L-cystathionine, appear to have no inhibitory effect on the enzyme, hydrogen sulfide may inhibit the enzymatic reaction of *S. gordonii* Lcd. Taken together, it is possible that Lcd from strains of the anginosus group is much more resistant to hydrogen sulfide than that from *S. gordonii*. The primary sequences of the Lcd proteins were more than 70% identical between *S. constellatus* or *S. anginosus* and *S. gordonii*. Those results suggest that the difference in the substrate specificity possibly depends on minor differences in amino acid sequences. It would be interesting to know what structure determines the substrate specificity among such closely related enzymes.

The effects of inhibitors on the *S. constellatus* Lcd activity were comparable to those reported previously for cystathionine lyases in *L. lactis* (18) and *Bordetella avium* (10). These enzymes are strongly inhibited by carbonyl reagents but are not inhibited by EDTA, indicating that metal ions are not required for their activity. The sulfhydryl reactive reagent iodoacetic acid caused marked inhibition of the enzyme. Five Lcd proteins of *S. constellatus* strains used in this study contained three cysteinyl residues (at positions 167, 189, and 254). In contrast, the last residue (i.e. Cys-254) was substituted by Tyr in the protein of *S. constellatus* IMU106, although the first two were conserved. These data suggest that oxido-reaction of -SH groups at positions 167 and/or 189 may be involved in catalysis.

Hydrogen sulfide, a toxic gas with the smell of rotten eggs, is one of the predominant volatile sulfur compounds in periodontal pockets (23). Moreover, it was recently reported that the gas is associated with endotoxin-induced inflammation (17) and apoptosis (35). Thus, the high production of hydrogen sulfide in periodontal pockets may be the result of the βC-S lyases of the anginosus group strains, many of which are found in subgingival plaque (8). Interestingly, Okayama et al. (22) reported that the majority of bacteria isolated from abscesses that were experimentally formed by the injection of native dental plaque into mice were of the anginosus group of streptococci, which are usually minor members of induced plaque samples. Taken together, it is possible that the high capacity of the anginosus group to produce hydrogen sulfide is associated with abscess formation. This hypothesis may be supported by a report that detected the members of the

anginosus group of streptococci in a gas-containing cystic mass in four of nine deep neck abscesses by computed tomography (9). However, critical evidence of a relationship between the high production of hydrogen sulfide by this group of bacteria and abscess formation is lacking. Persson et al. (24) reported that incubation of intact cells of strains belonging to the anginosus group of streptococci with L-cysteine did not result in high production of hydrogen sulfide, suggesting that this group of bacteria, like other oral gram-negative bacteria, may use peptides more efficiently than free amino acids (31).

We recently found that two of six *S. intermedius* strains, which belong to the anginosus group of streptococci, have an extremely low capacity to produce hydrogen sulfide from L-cysteine (S. Ito, T. Sasaki & Y. Yoshida, unpublished data). It would be interesting to evaluate the capacity for abscess formation using those strains. Additional studies are necessary to obtain precise information on the virulence of the anginosus group of streptococci.

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