Identification and molecular analysis of βC–S lyase producing hydrogen sulfide in *Streptococcus intermedius*

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Hydrogen sulfide (H_2S) is a toxic gas that induces the modification and release of haemoglobin in erythrocytes; however, it also functions in methionine biosynthesis in bacteria. βC–S lyase, encoded by the *lcd* gene, is responsible for bacterial H_2S production through the cleavage of L-cysteine. In this study, 26 of 29 crude extracts from reference and clinical strains of *Streptococcus intermedius* produced H_2S from L-cysteine. The capacities in those strains were not higher than those in strains of the other anginosus group of streptococci, *Streptococcus anginosus* and *Streptococcus constellatus*, but were much greater than those in strains of *Streptococcus gordonii*, which is known to have an extremely low capacity for H_2S production. Incubation of the remaining three extracts with L-cysteine did not result in H_2S production. Sequence analysis revealed that the *lcd* genes from these three strains (*S. intermedius* strains ATCC 27335, IMU151 and IMU202) contained mutations or small deletions. H_2S production in crude extracts prepared from *S. intermedius* ATCC 27335 was restored by repairing the *lcd* gene sequence in genomic DNA. The kinetic properties of the purified recombinant protein encoded by the repaired *lcd* gene were comparable to those of native proteins produced by H_2S-producing strains, whereas the truncated protein produced by *S. intermedius* ATCC 27335 had no enzymic activity with L-cysteine or L-cystathionine. However, real-time PCR analysis indicated that the *lcd* gene in strains ATCC 27335, IMU151 and IMU202 is transcribed and regulated in a manner similar to that in the H_2S-producing strain.

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INTRODUCTION

Micro-organisms require sulfur for the production of cysteine and methionine, which are essential for protein biosynthesis. Sulfur is also a constituent of several other indispensable cellular components, including thiamine, biotin, lipoic acid and coenzyme A. In general, sulfur is assimilated via the sulfate pathway, which involves the reduction of sulfate (SO_4^{2-}) to sulfide (S^{2-}) and the subsequent incorporation of S^{2-} into cysteine. Cysteine serves as a sulfur donor in the biosynthesis of methionine through the formation of homocysteine (Fig. 1). There are two alternative pathways for homocysteine biosynthesis: the trans-sulfuration pathway, in which cystathionine γ-synthase and cystathionine β-lyase transfer a sulfur atom from cysteine to homocysteine via cystathionine, a thioester intermediate, and the direct sulfhydrylation pathway, in which sulfur from hydrogen sulfide (H_2S) is directly fixed with *O*-acetylhomoserine or *O*-succinylhomoserine by acylhomoserine sulfhydrylase (Fig. 1). Ultimately, homocysteine is converted to methionine by methionine synthase (Soda, 1987). Enteric bacteria, such as *Escherichia coli*, use the trans-sulfuration pathway (Smith, 1971), whereas organisms such as *Saccharomyces cerevisiae* (Thomas & Surdin-Kerjan, 1997), *Rhizobium etli* (Tate *et al.*, 1999), *Pseudomonas aeruginosa* (Foglino *et al.*, 1995) and *Leptospira meyeri* (Belfaiza *et al.*, 1998) use the direct sulfhydrylation pathway.

We previously reported that both of these pathways function in *Streptococcus anginosus* (Yoshida *et al.*, 2003b). In the biosynthesis of homocysteine, the protein encoded by the *cgs* gene functions as a cystathionine γ-synthase in the trans-sulfuration pathway and as an acylhomoserine sulfhydrylase in the direct sulfhydrylation pathway (Fig. 1). In contrast, the *lcd* gene, which is co-

Abbreviations: PLP, pyridoxal 5′-phosphate; GST, glutathione *S*-transferase.

The GenBank/EMBL/DDBJ accession numbers for the *lcd* sequences of *S. intermedius* strains ATCC 27335, IMU202, IMU151, IMU105, IMU122, IMU201 and DP102 are AB381917, AB381918, AB381919, AB381920, AB381921, AB381922 and AB381923, respectively.

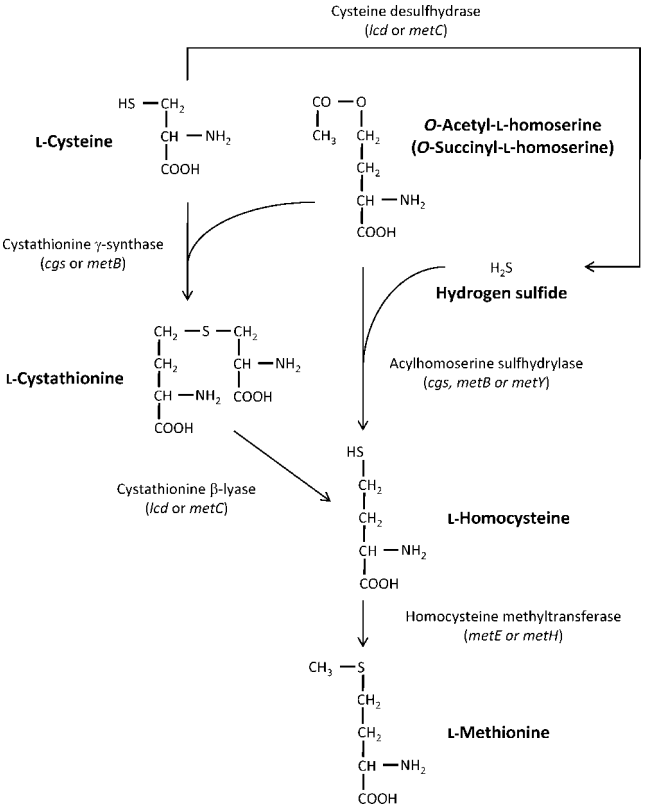

Fig. 1. Two pathways for the biosynthesis of methionine in bacteria. Genes encoding enzymes are indicated in parentheses. *O*-Acetyl-L-homoserine can be replaced by *O*-succinyl-L-homoserine.

transcribed with *cgs* as an operon, encodes βC–S lyase, also known as cystathionine β-lyase. This pyridoxal-5′-phosphate (PLP)-dependent enzyme catalyses the α,β-elimination of L-cysteine and L-cystathionine to produce H_2S and homocysteine, respectively (Yoshida *et al.*, 2002). βC–S lyase shows a high H_2S-producing capacity in *S. anginosus* and *Streptococcus constellatus* (Yoshida *et al.*, 2008), both of which belong to the anginosus group streptococci with *Streptococcus intermedius* (Kawamura *et al.*, 1999; Whiley *et al.*, 1990; Whiley & Beighton, 1991), whereas homologues from other oral streptococci, such as *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus salivarius*, are able to produce homocysteine from L-cystathionine, but are almost unable to form H_2S from L-cysteine (Yoshida *et al.*, 2003a). Hence, it appears that *S. anginosus* and *S. constellatus* use both the trans-sulfuration and direct sulfhydrylation pathways, whereas other streptococci that have been tested use only the former pathway. However, it is unclear whether the capacity to produce large amounts of H_2S is also common to the remaining species of the anginosus group (e.g. *S. intermedius*).

For this reason, we conducted molecular and enzymic studies of βC–S lyase associated with production of H_2S and homocysteine in the methionine biosynthetic pathway of *S. intermedius*. The *lcd* gene encoding βC–S lyase in *S. intermedius* strains and its products were characterized. The effects of *lcd* gene disruption, which were identified in the course of this study, were also characterized in three strains.

METHODS

Bacterial strains, culture conditions and genetic methods. The strains used in this study are listed in Table 1. The clinical isolates, which are indicated by the prefix IMU, were collected from clinical specimens submitted for culture to the Central Clinical Laboratory, Iwate Medical University Hospital (Morioka, Japan). Each IMU strain was isolated from a specimen collected from a distinct patient. The strains were identified as *S. intermedius* by PCR as previously described (Takao *et al.*, 2004). The source of other clinical isolates is described elsewhere (Whiley & Beighton, 1991; Nagamune *et al.*, 2000). The streptococci were grown anaerobically in brain heart infusion (BHI; Difco) broth at 37 °C. When required, spectinomycin or erythromycin was used to supplement the media at 250 or 10 μg ml^{-1}, respectively. *E. coli* strains DH5α (Invitrogen) and BL21 (Promega) were used for DNA manipulation and protein purification, respectively, and were grown aerobically at 37 °C in 2 × YT broth or agar (Difco), which was supplemented with 20 μg chloramphenicol ml^{-1}or 100 μg ampicillin ml^{-1} as required for maintenance of the plasmids.

Cloning and sequencing of the *lcd* genes. PCR fragments (~1.3 kb) containing the *lcd* gene from the *S. intermedius* strains ATCC 27335, IMU105, IMU122, IMU201, IMU202, IMU151 and DP102 were amplified using primers specific for the flanking regions of *lcd* in *S. anginosus* and *S. constellatus* (Yoshida *et al.*, 2008). Each amplified fragment was then cloned into pMCL210 (Nakano *et al.*, 1995) and sequenced using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequences were assembled and analysed using Vector NTI software (Invitrogen).

Preparation of crude enzyme extracts. Crude enzyme extracts were obtained as previously described (Yoshida *et al.*, 2002). Briefly, each streptococcal strain was grown in BHI to an OD_{600} of approximately 1.0, which corresponded to late exponential phase for all the strains. The cells were then harvested from 200 ml of culture and washed three times with cold PBS (0.12 M NaCl, 0.01 M Na_2HPO_4 and 5 mM KH_2PO_4, pH 7.5). A 500 μl aliquot of the cell suspension was then transferred to a screw-cap microcentrifuge tube containing 0.5 g of 0.1–0.15 mm-diameter glass beads. After vortexing the cells with the beads ten times for 30 s at 1 min intervals, the supernatant was centrifuged. The protein concentration in the extracts was then determined using a protein assay reagent (Bio-Rad) with BSA as a standard. After the addition of an equal volume of 80 % (v/v) glycerol, the samples were stored at −20 °C.

Repair of the *lcd* pseudogene in *S. intermedius* ATCC 27335. To obtain an *S. intermedius* ATCC 27335 mutant strain containing a repaired *lcd* gene, we initially constructed the mutant strain KO100, in which the entire region of the *lcd* pseudogene was replaced by a spectinomycin-resistance gene (*spc*). The intermediate mutant strain was produced to avoid unexpected recombination in the next step. The DNA fragment used for transformation was prepared by overlap extension PCR (Horton *et al.*, 1989), as previously described (Yoshida *et al.*, 2005). Briefly, each reaction mix contained KOD Hot Start DNA Polymerase (Toyobo), a template consisting of three overlapping PCR fragments (the upstream gene targeting sequence, the *spc* gene and the downstream gene targeting sequence) and primers

Table 1. *S. intermedius* strains used in this study

Strain	Isolation source/description	Reference
S. intermedius IMU105	Nasal swab	This study
S. intermedius IMU122	Tonsil abscess	This study
S. intermedius IMU136	Tonsil abscess	This study
S. intermedius IMU151	Blood	This study
S. intermedius IMU166	Dental abscess	This study
S. intermedius IMU168	Spinal puncture	This study
S. intermedius IMU169	Thoracic cavity abscess	This study
S. intermedius IMU201	Dental plaque	This study
S. intermedius IMU202	Dental plaque	This study
S. intermedius UNS46	Liver abscess	Whiley & Beighton (1991)
S. intermedius UNS38	Brain abscess	Whiley & Beighton (1991)
S. intermedius UNS32	Liver abscess	Whiley & Beighton (1991)
S. intermedius UNS42	Liver abscess	Whiley & Beighton (1991)
S. intermedius UNS35	Brain abscess	Whiley & Beighton (1991)
S. intermedius A4676	Brain abscess	Whiley & Beighton (1991)
S. intermedius HW13	Umbilical cord	Whiley & Beighton (1991)
S. intermedius F458s	Abdominal mass	Whiley & Beighton (1991)
S. intermedius HW69	Brain abscess	Whiley & Beighton (1991)
S. intermedius DP102	Dental abscess	Whiley & Beighton (1991)
S. intermedius DP101	Dental abscess	Whiley & Beighton (1991)
S. intermedius GN472	Dental plaque	Whiley & Beighton (1991)
S. intermedius E691	Eye	Whiley & Beighton (1991)
S. intermedius 2Q	Brain abscess	Whiley & Beighton (1991)
S. intermedius PC7466	Dental plaque	Whiley & Beighton (1991)
S. intermedius AC4720	Dental plaque	Whiley & Beighton (1991)
S. intermedius PC574	Dental plaque	Whiley & Beighton (1991)
S. intermedius AC800	Dental plaque	Whiley & Beighton (1991)
S. intermedius AC5803	Dental plaque	Whiley & Beighton (1991)
S. intermedius ATCC 27335	Unknown	Type strain
S. intermedius KO100	*S. intermedius* ATCC 27335 containing *spc* in place of the disrupted *lcd*	This study
S. intermedius KO101	*S. intermedius* KO100 containing the repaired *lcd* in place of *spc*	This study

complementary to the 5′ end of the upstream gene targeting sequence and the 3′ end of the downstream gene targeting sequence. Individual fragments were prepared by the amplification of *spc* from pDL278 (LeBlanc *et al.*, 1992) and targeting sequences from *S. intermedius* ATCC 27335 genomic DNA using appropriately designed primers. Next, the *spc* gene in strain KO100 was replaced with the repaired *lcd* linked to the erythromycin-resistance cassette (*ermAM*). Transforming DNA was prepared by three rounds of overlap extension PCR: the first to link the upstream gene targeting sequence to the repaired *lcd*, the second to link *ermAM* to the downstream gene targeting sequence and the third to link the two constructs together. The primers used to amplify these fragments were designed to create complementarity between the constructs. The repaired *lcd* was also amplified by overlap extension PCR using two complementary primers with an inserted cytosine. The *ermAM* cassette and the gene targeting sequences were prepared from pKSerm2 (Lunsford, 1995) and *S. intermedius* ATCC 27335 genomic DNA, respectively.

The transformation of *S. intermedius* was performed as previously described (Lunsford, 1995) with minor modifications. Briefly, an overnight Todd–Hewitt broth (THB) culture was diluted 1 : 20 in THB containing 5 % (v/v) heat-inactivated horse serum (THB-HS). Following incubation for 2 h at 37 °C, the resulting culture was again diluted 1 : 20 in fresh THB-HS and incubated for 2 h at 37 °C to obtain early-exponential-phase competent cells. Transformation reactions containing 50 µl competent cells, 450 µl THB-HS, 2 µg transforming DNA and 50 ng competence-simulating peptides (Havarstein *et al.*, 1997) were incubated for 2 h at 37 °C prior to plating on BHI agar containing 5 % (v/v) heat-inactivated horse serum and spectinomycin or erythromycin.

Purification of recombinant βC–S lyase. Recombinant Lcd proteins from the *S. intermedius* strains ATCC 27335, IMU201 and KO101 were purified using the expression vector pGEX-6P-1 (GE Healthcare), as previously described (Yoshida *et al.*, 2002). Briefly, each *lcd* gene was amplified by PCR using primers designed to incorporate a *Bam*HI site at the 5′ end and a *Sal*I or *Xho*I site at the 3′ end of each segment. Following amplification, the products were digested with the appropriate restriction enzymes and ligated into pGEX-6P-1, juxtaposing each *lcd* fragment downstream of the coding sequence for glutathione *S*-transferase (GST) and a PreScission protease (GE Healthcare) cleavage site. The recombinant *E. coli* clones were grown in 2 × YT broth containing ampicillin to an OD_{600} of about 0.8, and IPTG was added to a final concentration of 0.5 mM. The cells were harvested 2 h after induction, washed with PBS, and then lysed by ultrasonication. After centrifugation at 15 000 ***g*** for 30 min at 4 °C, the lysates were incubated with glutathione-Sepharose 4B beads (GE Healthcare). After extensive washing, the

beads were treated with PreScission protease to recover the purified protein. The protein concentration was determined using Bio-Rad protein assay reagent, and the purity of the samples was verified by SDS-PAGE.

Visualization of enzymic activity. L-Cysteine desulfhydrase activity of crude enzyme extracts was visualized using non-denaturing polyacrylamide gels as previously described (Claesson *et al.*, 1990). The samples were electrophoresed at 10 mA per gel at 4 °C for 2.5 h on 12.5 % (w/v) resolving (pH 8.8) and 3 % (w/v) stacking (pH 6.5) polyacrylamide gels. After electrophoresis, the gel was incubated in visualizing solution (100 mM triethanolamine.HCl, pH 7.6, 10 μM PLP, 0.5 mM bismuth trichloride, 10 mM EDTA and 5.0 mM L-cysteine) at 37 °C to allow detection of the enzyme's position.

Enzyme activity assay. The level of activity of the βC–S lyases was examined by measuring the rate of formation of H_2S or pyruvate. To estimate H_2S production, a methylene blue formation assay was performed following the method of Schmidt (1987). Briefly, the reaction mixture contained the following reagents 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 10 min of incubation at 37 °C, the reaction was terminated by the addition of 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 examined spectrophotometrically at 670 nm using a molar absorption coefficient of 28.5×10^6 M^{-1} cm^{-1}.

Pyruvate formation was measured as previously described (Soda, 1968). The assay was carried out in a reaction mixture (100 μl) containing 50 mM potassium phosphate buffer (pH 7.6), 1 nmol PLP, 170 ng (for L-cysteine) or 40 ng (for L-cystathionine) purified enzyme and various amounts of each substrate. After 2 min incubation at 37 °C, the reaction was terminated by the addition of 50 μl 4.5 % (v/v) trichloroacetic acid. The reaction mixture was then centrifuged and 100 μl of the supernatant was added to 300 μl 0.67 M sodium acetate (pH 5.2) containing 0.017 % (w/v) 3-methyl-2-benzothiazolinone hydrazone. After incubation at 50 °C for 30 min, the absorbance at 335 nm was determined. The amount of pyruvate was calculated from a standard curve prepared using crystalline sodium pyruvate. The kinetic parameters were computed from a Lineweaver–Burk transformation (V^{-1} versus S^{-1}) of the Michaelis–Menten equation, where V (μmol min^{-1} mg^{-1}) represented the formation of pyruvate and S (mM) was the concentration of each substrate. All values are reported as the means ± SD of three independent experiments.

Real-time PCR analysis. Each strain was grown in 40 ml BHI to an OD_{600} of about 1.0 (late exponential phase). Total RNA was then isolated from the harvested cells using FastPrep Blue tubes (Bio 101). Contaminating DNA was eliminated by digestion with RNase-free DNase (Takara Bio). RNA (10 ng) was reverse transcribed into single-stranded cDNA using PrimeScript Reverse Transcriptase (Takara Bio) according to the manufacturer's instructions. Real-time quantitative PCR amplification, detection and analysis were performed using the Thermal Cycler Dice RealTime System (Takara Bio) with Power SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR was carried out in 25 μl reaction mixtures (1 × Power SYBR Green PCR Master Mix, 22.5 pmol of each forward and reverse primers and 2.5 μl template). The reaction conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. At the end of each run, a dissociation protocol (95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s) was performed to ensure that non-specific PCR products were absent. Each primer was designed using Primer Express software (version 3.0; Applied Biosystems). The amount of 16S rRNA was used as an internal standard to normalize for the amount of total RNA in each sample. The primers used to amplify the *lcd* gene were 5′-GGA CTT TGA AGT CAT GCC TGA AGT A-3′ and 5′-CAC TGC TTG GAC TAG CTC ATC ACT-3′, while those used for 16S rRNA were 5′-GGA CTT TGA ACT CAT GCC TGA AGT A-3′ and 5′-CAC TGC TTG GAG TAG CTC ATC ACT-3′. To estimate the initial amounts of template in each sample, serial real-time PCR was performed using purified streptococcal genomic DNA. For each gene, a standard curve was plotted using the log of the initial quantity of template against the threshold cycle (i.e. the cycle at which the fluorescence rose above the background level). In this way, differences in primer efficiency could be accommodated. The data were obtained from three independent experiments.

High-performance liquid chromatography (HPLC). Production of homocysteine or cysteine from cystathionine by βC–S lyase was determined on a reversed-phase column using HPLC. The reaction mixture contained the following reagents in a final volume of 100 μl: 50 mM potassium phosphate buffer (pH 7.6), 10 μM PLP, 2 mM L-cystathionine and 0.4 μg purified βC–S lyase from *S. intermedius* IMU201. After the mixture had been incubated for 6 h at 37 °C, the enzyme was removed using a Microcon YM-10 filter (10 kDa cutoff; Amicon). The ultrafiltration product was determined after derivatization with dansyl chloride as described by Tapuhi *et al.* (1981). An aliquot (20 μl) of the sample was injected onto an XTerra RP_{18} column (4.6 × 150 mm; Waters). A linear gradient (60–80 %) of methanol in distilled water containing 0.6 % (v/v) glacial acetic acid and 0.008 % (v/v) triethylamine was used at a flow rate of 1.0 ml min^{-1} at 40 °C. Excitation and emission wavelengths of 350 and 530 nm, respectively, were used.

Statistical analysis. All data were analysed using Student's *t*-tests. A P value of <0.05 was considered significant.

RESULTS AND DISCUSSION

H_2S production from L-cysteine in crude enzyme extracts prepared from *S. intermedius*

To examine H_2S production in *S. intermedius* strains, crude enzyme extracts were obtained from one reference and 28 clinical strains of *S. intermedius*. H_2S production from L-cysteine was generally lower than that of *S. anginosus* and *S. constellatus* (Fig. 2). In contrast, 26 of the 29 extracts showed a greater capacity for H_2S production than in *S. gordonii* strains. Incubation of the three remaining crude extracts, which were derived from one reference and two clinical strains (strains ATCC 27335, IMU151 and IMU202), with L-cysteine did not produce H_2S. These results were unexpected, because crude extracts from more than 25 streptococcal strains grown in BHI were able to produce H_2S from L-cysteine. There are four possible explanations for the lack of detectable H_2S production: (1) the existence of truncated protein encoded by a mutant *lcd* gene, (2) no (or extremely low) transcription of *lcd*, (3) no (or extremely low) enzymic activity of the purified Lcd for L-cysteine or (4) no (or extremely low) translation from mRNA of the *lcd* gene. These possibilities were evaluated in the following experiments.

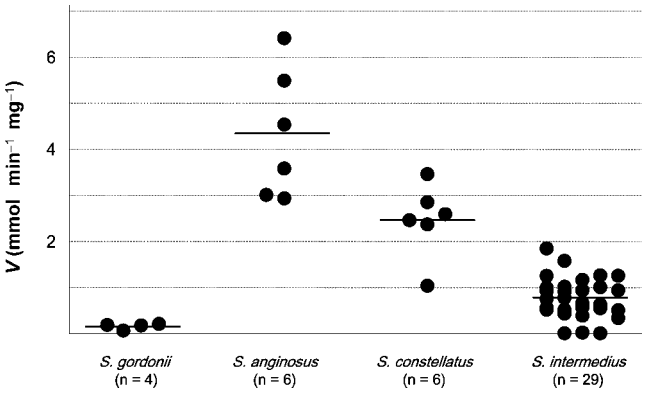

Fig. 2. H_2S production in crude enzyme extracts from streptococcal strains. Each extract was incubated with L-cysteine (1 mM) for 10 min. The data are the means of three independent experiments. Bars indicate the mean level of H_2S formation in each group. Activity data for *S. anginosus*, *S. constellatus* and *S. gordonii* (Yoshida *et al.*, 2008) were compared to those for *S. intermedius*.

Molecular analysis of the *lcd* gene encoding βC–S lyase in *S. intermedius*

To analyse the genetic basis of H_2S production in *S. intermedius*, DNA fragments containing the *lcd* gene in seven *S. intermedius* strains, including four H_2S-producing (IMU105, IMU122, IMU201 and DP102) and three non-producing (ATCC 27335, IMU151 and IMU202) strains were sequenced and analysed. The *lcd* homologues from the H_2S-producing strains were 1164 bp long, which is identical to the length of the *lcd* genes in *S. anginosus* and *S. constellatus*. Database analysis revealed that the deduced amino acid sequences from these *S. intermedius* strains had high identity to those from 12 strains of *S. anginosus* and *S. constellatus* (90.5–94.1 %) (Yoshida *et al.*, 2008). They also showed moderate sequence similarity with the amino acid sequences of four *S. gordonii* strains (75.0–75.5 %) (Yoshida *et al.*, 2008). The Lcd protein from *S. intermedius* ATCC 27335 was shorter than those of the other strains, and primary sequence alignment revealed that 119 amino acids were missing from the C terminus of the *S. intermedius* ATCC 27335 Lcd protein. This mutation involved the deletion of a cytosine at position 809 in the *lcd* gene, which effectively changed a TCA (Ser) codon to a TAA terminator. A different mutation (the deletion of an adenine and a thymine at positions 180 and 181, which created a TGA terminator 13 bp further downstream) was, surpisingly, present in the *lcd* gene from both strain IMU151 and strain IMU202. Consequently, their deduced Lcd sequences lacked 47 amino acids at the N terminus, compared with the intact protein. Moreover, 1161 of the 1162 bp in these two truncated *lcd* genes were identical. These findings might suggest that these two strains had acquired the same truncated gene by lateral transfer. The presence of such mutations also suggests that selective pressure to maintain the complete gene may have been removed. If this were true, *S. intermedius* strains with disrupted *lcd* genes could be auxotrophs for methionine. To test this hypothesis, we attempted to grow these strains of *S. intermedius* in two different types of previously described chemically defined media (Socransky *et al.*, 1985; Terleckyj *et al.*, 1975), with or without methionine. However, even *S. intermedius* strains containing an active *lcd* gene failed to grow in these media. Thus, the effect of *lcd* disruption on auxotrophy in *S. intermedius* remains to be clarified.

Repair of the truncated *lcd* in *S. intermedius* ATCC 27335

We suspected that *S. intermedius* ATCC 27335 would have the capacity to produce H_2S from L-cysteine following repair of the *lcd* pseudogene. Gene repair was accomplished in two steps (Fig. 3a). The repaired *lcd* contained cytosine at position 809, which was commonly identified in the other sequenced *lcd* genes of *S. intermedius* strains IMU105, IMU122, IMU201, IMU202, IMU151 and

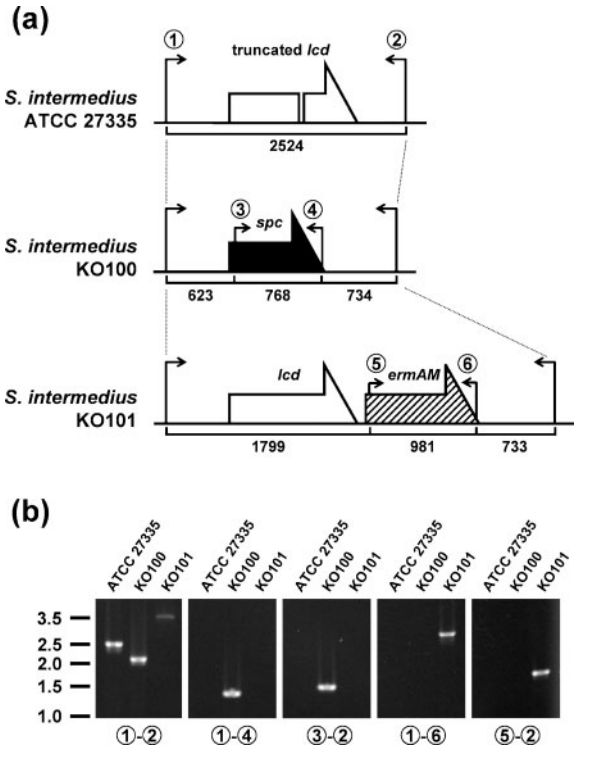

Fig. 3. Construction of a derivative of *S. intermedius* ATCC 27335 containing a repaired *lcd* gene. Each circled number indicates a primer used for verification. (a) Chromosomal gene arrangement in the parental and mutant strains. The size of each fragment is shown in base pairs. (b) Verification of the mutants by agarose gel electrophoresis. Each fragment was PCR amplified using the primers indicated. The positions of DNA size standards (kb) are shown.

DP102. Integration of the overlap extension PCR products at the expected location in the chromosome was confirmed by amplification of the upstream and downstream boundaries of the insertion using primers specific for flanking sequences that were extraneous to those used for gene targeting (Fig. 3b). Repair of the *lcd* gene in strain KO101 was confirmed by sequencing of a PCR product amplified from the strain's genomic DNA.

Incubation of crude extracts from strain KO101 with L-cysteine resulted in H_2S production, indicating that the capacity of strain ATCC 27335 to produce H_2S was restored by repair of the *lcd* gene (Fig. 4a). The rate of L-cysteine degradation in crude extracts prepared from strain KO101 was lower than that in extracts of *S. anginosus* and *S. constellatus*, but higher than that in extracts prepared from *S. gordonii* and similar to the rate in other *S. intermedius* strains (Fig. 2). Furthermore, the restoration was confirmed by *in situ* staining, in which H_2S is allowed to react with bismuth to produce an insoluble product that forms brown bands on non-denaturing gels. A single band associated with the production of H_2S was detected in crude extracts prepared from strain KO101, whereas strains ATCC 27335 and KO100 showed no detectable bands (Fig. 4b). These findings demonstrate that no other enzymes are responsible for the production of H_2S and that the frameshift in the *lcd* gene abolished H_2S production in ATCC 27335. It is possible that repair of the *lcd* genes in strains IMU151 and IMU202 might also restore H_2S production.

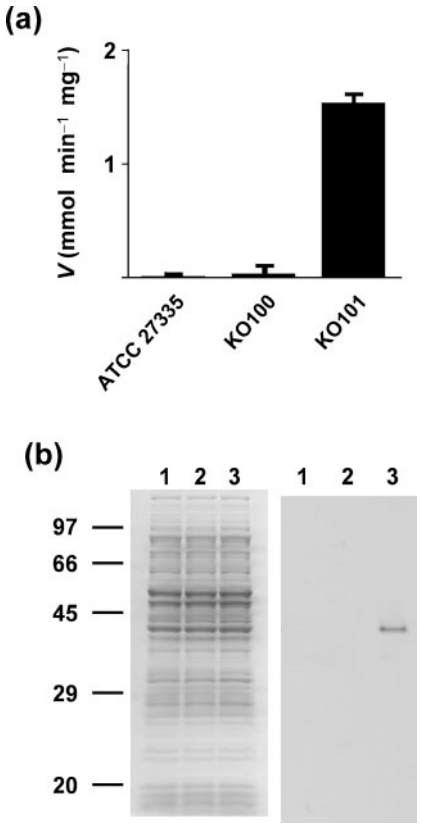

Fig. 4. Restoration of H_2S production by repair of the *lcd* gene in *S. intermedius* ATCC 27335. (a) H_2S production from 1 mM L-cysteine with crude extracts. The values represent the means ± SD of three independent experiments. (b) Electrophoresis of crude extracts of the parental and mutant strains of *S. intermedius*. Left panel: the samples (45 μg) were subjected to SDS-PAGE, and the gel was subsequently stained with Coomassie brilliant blue. Right panel: the samples (45 μg) were subjected to non-denaturing PAGE, and enzymic activity was visualized. Lanes: 1, *S. intermedius* ATCC 27335 cell lysate; 2, *S. intermedius* KO100 cell lysate; 3, *S. intermedius* KO101 cell lysate. The positions of molecular mass markers (kDa) are shown.

Quantification of *lcd* transcription in *S. intermedius*

The fact that we were able to restore H_2S production by *lcd* gene repair suggests that *lcd* transcription was not interrupted in strain ATCC 27335. Real-time PCR analysis was used to evaluate the relative abundance of *lcd*-bearing transcripts among strains ATCC 27335, IMU151 and IMU202 (with an inactive *lcd* gene), KO101 (with a repaired *lcd* gene) and IMU201 (with an active *lcd* gene). To estimate the initial quantity of template in each sample, tenfold serial dilutions of genomic DNA were prepared to generate a standard curve using primers to amplify *lcd* (Fig. 5a) and 16S rRNA (data not shown). The purity of the amplified products was assessed by dissociation curve analysis (Fig. 5b) and electrophoresis on 1.8 % (w/v) agarose gels (data not shown). As shown in Fig. 5(c), these strains did not significantly differ in *lcd* transcript abundance, suggesting that the mutation in *lcd* had no effect on gene expression in strain ATCC 27335.

Purification and enzymic characterization of recombinant Lcd

To characterize the enzymic activity of the *lcd* products, recombinant Lcds from *S. intermedius* strains ATCC 27335, KO101 and IMU201 were purified. Each purified protein was obtained by cleavage of the GST-fusion protein bound to glutathione-Sepharose 4B. SDS-PAGE was used to assess the purity of each recombinant Lcd (Fig. 6). The observed size of the proteins was consistent with their predicted molecular masses (31 kDa for ATCC 27335 and 44 kDa for KO101 and IMU201). The reason why the sample from ATCC 27335 contained unexpected faint bands is unknown. The same purification method failed to isolate the other truncated *lcd* product of *S. intermedius* IMU202, whose amino acid sequence was identical to that of *S. intermedius* IMU151. Since the GST-Lcd fusion protein of strain IMU202 was largely produced in *E. coli* cells, PreScission protease might not recognize the core amino acid sequence for cleavage between GST and Lcd. Indeed, substrate recognition and cleavage are likely to be dependent not only upon primary structural signals, but

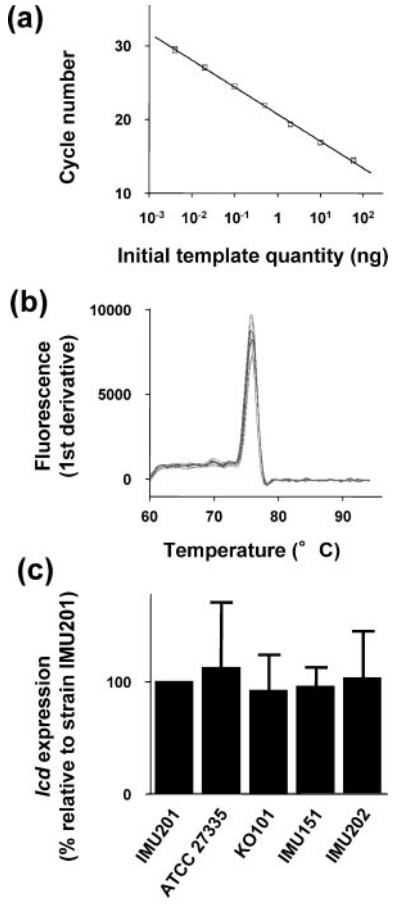

Fig. 5. Real-time quantitative PCR analysis. (a) Standard curve. The reaction mixture contained the primers used for *lcd* amplification and genomic DNA extracted from *S. intermedius* ATCC 27335. The standard curve is a plot of the initial template quantity (*x*-axis) versus the threshold cycle (R^2=0.999). (b) Dissociation curve analysis of the real-time PCR products used to prepare the standard curve shown in (a). The plot was based on the first derivative of the fluorescence reading. The specific PCR products melted at 77±1 °C. (c) Comparison of *lcd* expression among *S. intermedius* IMU201, ATCC 27335, KO101, IMU151 and IMU202. The amount of *lcd* cDNA was determined and normalized against the amount of 16S rRNA cDNA following reverse transcription of total RNA. The expression of *lcd* in strains ATCC 27335, KO101, IMU151 and IMU202 is indicated relative to that in strain IMU201. The data are the means±SD of three experiments; the cDNA used in each experiment was generated from independently isolated RNA.

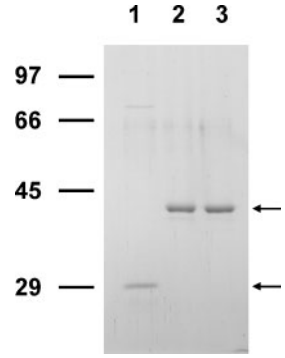

Fig. 6. SDS-PAGE analysis of recombinant purified *lcd* products from the *S. intermedius* strains. The samples were subjected to SDS-PAGE and the gel was stained with Coomassie brilliant blue. Lanes: 1, recombinant Lcd from *S. intermedius* ATCC 27335; 2, recombinant Lcd from *S. intermedius* KO101; 3, recombinant Lcd from *S. intermedius* IMU201. The positions of molecular mass markers (kDa) are shown. Arrows indicate the 44 and 31 kDa bands of the predicted sizes.

also upon the secondary and tertiary structures of the fusion protein (manufacture's protocol). Alternatively, the GST-Lcd fusion protein might be insoluble due to formation of inclusion body in host cells.

Some βC–S lyases have been reported not only to cleave cystathionine to yield ammonia, pyruvate and homocysteine via α,β-elimination, but also to degrade cystathionine to ammonia, α-ketobutyrate and cysteine via α,γ-elimination (Alting *et al.*, 1995; Dobric *et al.*, 2000) To determine whether Lcd of *S. intermedius* catalyses α,γ-elimination, HPLC analysis was carried out (Fig. 7). Each amino acid was detected as a dansylated product. Incubation of Lcd from *S. intermedius* IMU201 with cystathionine resulted in production of homocysteine, while no production of cysteine was detected. These results demonstrated that Lcd from *S. intermedius* catalyses only α,β-elimination. Dansylhomocysteine was detected at 12.97 and 13.56 min in this condition. This may be due to the disulfide formation by oxidation of SH-compounds (Schulze & Neuhoff, 1976).

The breakdown of L-cysteine and L-cystathionine was monitored by assaying the production of pyruvate, which is a by-product of the reactions that degrade these substrates. The truncated Lcd of *S. intermedius* ATCC 27335 was unable to degrade L-cysteine or L-cystathionine (data not shown), suggesting that both the trans-sulfuration and direct sulfhydrylation pathways may be interrupted in this strain (Fig. 1). In contrast, the protein encoded by the repaired *lcd* in strain KO101 had a slightly higher capacity to degrade L-cysteine and L-cystathionine than the Lcd from strain IMU201, which contained an active *lcd*. The kinetic properties of the Lcds from *S. intermedius* KO101 and IMU201, which were calculated from Lineweaver–Burk plots, are summarized in Table 2. The K_m values of the Lcds from *S. intermedius* KO101 and IMU201 for L-cysteine (0.26 and 0.43 mM, respectively) were lower than those for the type strains of *S. anginosus* and *S. constellatus* (0.99 and 0.67 mM, respectively), indicating that the Lcd from *S. intermedius* has a high affinity for L-cysteine. In terms of the K_m value for L-cystathionine, the *S. intermedius* strains were comparable to *S. anginosus* and *S. constellatus* (0.41 and 0.71 mM, respectively). Thus, both

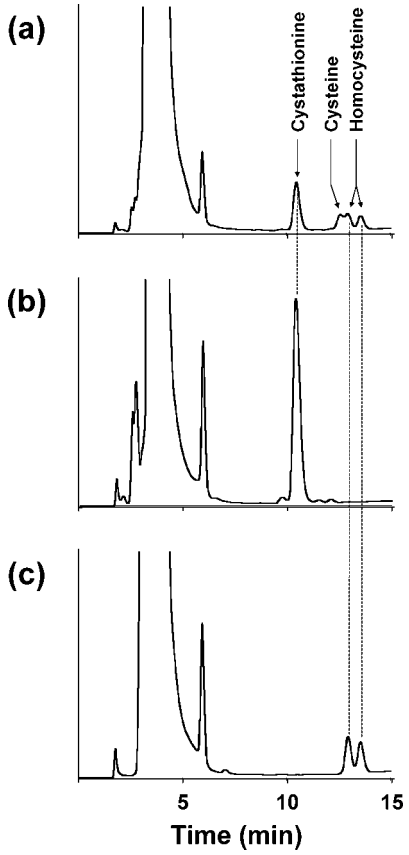

Fig. 7. Reversed-phase HPLC profiles of dansylated reaction products. (a) A mixture of pure standard chemicals. (b) Reaction products obtained with no enzyme (negative control). (c) Reaction products obtained with purified Lcd from *S. intermedius* IMU201. Peaks were identified by retention time.

the trans-sulfuration and direct sulfhydrylation pathways of methionine biosynthesis may be at work in *S. intermedius*. Kredich (1996) described that the direct sulfhydrylation pathway may be physically irrelevant due to the high concentration of sulfide required. In this regard, it is noteworthy that the βC–S lyase in *S. intermedius*, as well as those in *S. anginosus* and *S. constellatus*, was shown to have an extremely high capacity for H_2S production from L-cysteine compared to other oral streptococci, including *S. gordonii*, *S. oralis*, *S. mutans*, *S. sobrinus* and *S. salivarius* (Yoshida *et al.*, 2003a, 2008).

Concluding remarks

H_2S is widely known as a toxic gas with the smell of rotten eggs. It was recently discovered, however, that the gas is also associated with endotoxin-induced inflammation (Li *et al.*, 2005) and apoptosis (Yang *et al.*, 2004). Therefore, H_2S production in anginosus group streptococci may be associated with abscess formation, based on previous research showing that these bacteria are frequently encountered in suppurative infections in a variety of clinical sites, such as liver and brain abscesses (Gossling, 1988; Jacobs *et al.*, 1995; Molina *et al.*, 1991; Van der Auwera, 1985; Whiley *et al.*, 1992). In this regard, it is notable that the three *S. intermedius* strains containing a disrupted *lcd* gene (ATCC 27335, IMU202 and IMU151) were not isolated from abscesses. The type strain ATCC 27335 isolated some decades ago has been maintained since then in a laboratory environment. Indeed, there was no significant difference in growth among *S. intermedius* strains ATCC 27335, KO100 and KO101, when incubated in BHI, which appears to fulfil the nutritional requirements of the bacteria (data not shown). In contrast, two clinical strains with the disrupted *lcd* may occupy an ecological niche where methionine biosynthesis and/or H_2S production is unnecessary. However, critical evidence of a relationship between elevated H_2S production and abscess formation is lacking. Additional studies on abscess formation by H_2S-producing or non-producing strains are therefore necessary.

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Table 2. Kinetic properties of the *lcd* products from *S. intermedius* strains

Values are given as the means ± SD of three determinations.

	L-Cysteine		L-Cystathionine	
	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
S. intermedius KO101	0.26 ± 0.01	62 ± 0.01	0.74 ± 0.12	146 ± 6
S. intermedius IMU201	0.43 ± 0.06	61 ± 4.9	0.63 ± 0.25	91 ± 11

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