Oral Microbiology Immunology 2009: 24: 485–492
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Characterization of L-cysteine desulfhydrase from *Prevotella intermedia*

T. Yano[1,2], H. Fukamachi[2], M. Yamamoto[1], T. Igarashi[2]

[1]Department of Periodontology, Showa University School of Dentistry, Shinagawa-ku, Tokyo, Japan, [2]Department of Oral Microbiology, Showa University School of Dentistry, Shinagawa-ku, Tokyo, Japan

Yano T, Fukamachi H, Yamamoto M, Igarashi T. Characterization of L-cysteine desulfhydrase from Prevotella intermedia.
Oral Microbiol Immunol 2009: 24: 485–492. © 2009 John Wiley & Sons A/S.

Introduction: Hydrogen sulfide is responsible for lysis of red blood cells and is a major compound for oral malodor. To clarify the production mechanism of hydrogen sulfide in *Prevotella intermedia*, we found an L-cysteine desulfhydrase gene (*lcs*) homologue on the genome database of *P. intermedia* ATCC25611 and characterized its gene product.
Methods: The *lcs* gene homologue cloned into pGEX6p-1 vector was expressed in *Escherichia coli* and purified. Lcs activity was assayed by detection of the reaction products (hydrogen sulfide and pyruvate) or its derivatives from L-cysteine. Site-directed mutagenesis was used to convert an amino acid of the Lcs molecule.
Results: The purified *lcs* gene product catalysed the degradation of L-cysteine to pyruvate, ammonia, and hydrogen sulfide, indicating that the protein is L-cysteine desulfhydrase. The enzyme required pyridoxal 5′-phosphate as a cofactor, and it was highly active at pH 7.0 and completely inhibited by $ZnCl_2$. The K_m and V_{max} of the enzyme were 0.7 mM and 4.2 μmol/min/mg, respectively. Replacement of Tyr-59, Tyr-118, Asp-198, and Lys-233 with any of the amino acids resulted in the complete disappearance of Lcs activity, implying that these amino acids are essential for enzyme activity. In addition, hydrogen sulfide produced by this enzyme lysed sheep red blood cells and modified hemoglobin.
Conclusion: These results show the enzymatic properties of L-cysteine desulfhydrase from *P. intermedia* ATCC25611 and also suggest that the Lcs enzyme, which produces hydrogen sulfide from L-cysteine, is closely associated with the pathogenesis of *P. intermedia*.

Key words: hydrogen sulfide; L-cysteine desulfhydrase; *Prevotella intermedia*

Takeshi Igarashi, Department of Oral Microbiology, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
Tel.: + 81 3 3784 8166;
fax: + 81 3 3784 4105;
e-mail: igatakes@dent.showa-u.ac.jp

Accepted for publication July 9, 2009

Prevotella intermedia, a gram-negative, black-pigmented anaerobic rod, is frequently isolated from periodontal lesions associated with various forms of periodontal diseases such as chronic periodontitis, pregnancy gingivitis, and acute necrotizing ulcerative gingivitis (17, 20, 26, 33, 45). This bacterium produces a variety of virulence factors including adhesins, fimbriae, hemagglutinin, hydrolytic enzymes, and hemolysin (21, 31, 39, 42, 43) and may play a role in the initiation and development of periodontal disease (26, 33, 41, 45).

Like other periodontal pathogens, *P. intermedia* requires iron for growth; however, it lacks the ability to synthesize heme, an important growth factor for the bacterium (18, 28). Therefore, sufficient mechanisms to acquire iron are needed to survive and proliferate in the host. Hemoglobin or hemin derived from the hemolytic degradation of red blood cells is a major source of exogenous iron for the nutrient of bacterial growth. There are several reports that *P. intermedia* has hemolytic and hemagglutinating activities (4, 39, 42). Although a hemolysin activated by reductants from *P. intermedia* was partially purified and characterized (43) and a gene encoding multiple hemolytic domains from *P. intermedia* was cloned (3), the hemolytic substances and the mechanism of hemolysis by *P. intermedia* are still unknown.

P. intermedia is frequently detected from patients with not only periodontal diseases but also oral malodor (27, 44). Previous studies have suggested a high correlation between periodontal diseases and oral malodor (12, 36, 46). The major compounds contributing to oral malodor are volatile sulfur compounds (VSC),

including hydrogen sulfide, methyl mercaptan, and dimethyl sulfide (25), and these VSC have been found at relatively high levels in periodontal pockets (5, 11, 40). Several studies reveal that of these VSC, hydrogen sulfide induces lysis of red blood cells (RBCs) (29, 37, 47) and is highly toxic for mammalian cells (2). It is reported that genera such as *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Selenomonas*, *Veillonella*, *Peptostreptococcus*, and *Eubacterium* are the predominant producers of hydrogen sulfide (40). Genetic and biochemical analyses have shown that hydrogen sulfide is produced by a bacterial enzyme. Until now, this type of enzyme has been identified, cloned, and characterized in oral bacteria such as *Treponema denticola* (9), *Fusobacterium nucleatum* (15), and *Streptococcus anginosus* (47). These studies demonstrate that hydrogen sulfide is produced from L-cysteine by L-cysteine desulfhydrase, which catalyses the α,β-elimination of L-cysteine to produce hydrogen sulfide, pyruvate, and ammonia. As *P. intermedia* is a predominant producer of hydrogen sulfide (40), the presence of the L-cysteine desulfhydrase-like enzyme in *P. intermedia* would be expected. Therefore, finding and analysing the enzyme participating in the production of hydrogen sulfide would promote the understanding of the acquisition mechanism of iron (hemoglobin or hemin) in *P. intermedia*.

In this study, we cloned the gene homologue encoding L-cysteine desulfhydrase in *P. intermedia* ATCC25611 and characterized its gene product. In addition, the hemolytic activity and hemoglobin modification by this enzyme were examined.

Materials and methods

Bacterial strains and culture conditions

P. intermedia ATCC25611 was grown anaerobically (10% CO_2, 10% H_2, 80% N_2) at 37°C in brain–heart infusion broth (Difco Laboratories, Detroit, MI) with hemin (5 μg/ml) and menadione (50 μg/ml). *Escherichia coli* BL21 was used as a plasmid host and cultured aerobically at 37°C in Luria–Bertani broth (Invitrogen, Carlsbad, CA).

DNA manipulation

Chromosomal DNA of *P. intermedia* ATCC25611 was extracted with a GenElute Bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO). Plasmid DNA was extracted with a Wizard miniprep purification kit (Promega, Madison, WI). Ligation of DNA was performed with a DNA Ligation Kit version 2 (TakaRa Bio, Shiga, Japan) and site-directed mutagenesis of the DNA fragments was carried out with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the supplier (22).

DNA sequence of L-cysteine desulfhydrase gene homologue of *P. intermedia* ATCC25611

The DNA fragment [1547 base pairs (bp) long] containing the L-cysteine desulfhydrase gene (*lcs*) homologue from *P. intermedia* ATCC25611 was amplified with the primer pair designed on the basis of the PI0305 sequence, which encodes putative L-cysteine desulfhydrase, on the *P. intermedia* strain 17 genome DNA database (http://www.oralgen.lanl.gov/oralgen/bacteria/pintnew/). The primer sequences were as follows: forward, 5′-GACC GTGCATTCGAAGACAG-3′ and reverse, 5′-CTAAGTATTTCTGAATTACAA-3′. Long and accurate polymerase chain reaction (LA-PCR) with LA *Taq* DNA polymerase (TakaRa Bio) was carried out to amplify the gene and Shuttle PCR was used as a condition of amplification, as recommended by the supplier (TakaRa Bio) (23). The amplified fragment (1547 bp) containing the *lcs* homologue (1170 bp) was cloned into pT7 Blue T-vector (Novagen, Madison, WI) and subcloned for DNA sequencing.

Cloning, expression, and purification of recombinant Lcs

The *lcs* gene homologue from *P. intermedia* ATCC25611 was amplified by LA-PCR with LA *Taq* DNA polymerase. PCR primers were designed on the basis of the nucleotide sequence of the *lcs* homologue from *P. intermedia* ATCC25611 determined in this study. The primer sequences were as follows: forward, 5′-GGG GAATTCATGGGAAAATATAATTTCGA CGAGC-3′ and reverse, 5′-GGGGTCGAC TTCACAGGCTTTCAACACGTTTACG-3′. The underline in each primer indicates the *Eco*RI and *Sal*I restriction sites, respectively. Shuttle PCR was used as a condition of amplification as recommended by the supplier (TakaRa Bio) (23). Briefly, the reaction mixture (50 μl) was denatured at 94°C for 1 min followed by 28 cycles of 20 s at 98°C for denaturation and 2 min at 68°C for annealing and extension. The amplified fragment (1170 bp) was cloned into a pGEX-6P-1 vector (GE Healthcare UK, Little Chalfont, UK) and the resulting plasmid was designated as pCDS1. The pCDS1 was transformed into *E. coli* BL21 cells and expressed. The recombinant Lcs protein was purified by the GST purification system according to the manufacturers' instructions (GE Healthcare).

Assay of L-cysteine desulfhydrase activity

L-Cysteine desulfhydrase activity was detected using three different assays.

Assay I: formation of methylene blue

The assay was carried out in a 1-ml reaction mixture containing 100 mM Tris–HCl (pH 7.0), 2.5 mM dithioerythritol, 10 μM pyridoxal-5′-phosphate (PLP), 1 mM L-cysteine, and enzyme. After incubation at 37°C, the reaction was terminated by the addition of 0.1 ml of 20 mM *N*′,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 0.1 ml of 30 mM $FeCl_3$ dissolved in 1.2 M HCl. After 20 min of incubation at room temperature, the formation of methylene blue was determined at an optical density at 670 nm (OD_{670}) using a molar extinction coefficient of 28.5×10^6 (15).

Assay II: formation of black precipitate from bismuth

The sample was suspended in a visualizing solution containing 100 mM triethanolamine–HCl (pH 7.0), 0.5 mM bismuth trichloride, 1% Triton X-100, 10 mM ethylenediaminetetraacetic acid, 10 μM PLP, and 5 mM L-cysteine. The bismuth in this solution reacts with sulfide, forming a black precipitate. This method was used to visualize the hydrogen sulfide-producing activity after non-denaturing polyacrylamide gel electrophoresis (native–PAGE) as described previously (47). After electrophoresis of the enzyme, the gel was soaked in the visualizing solution at 37°C. A black precipitate formed at the position of the enzyme producing hydrogen sulfide in the gel.

Assay III: formation of pyruvate

The assay was carried out with 200 μl of 100 mM Tris–HCl (pH 7.0) containing 10 μM PLP and 1 mM L-cysteine. The enzyme was added to start the reaction. After incubation for 20 min at 37°C, the reaction was terminated by adding 100 μl of 4.5% trichloroacetic acid. The suspension was centrifuged, and 250 μl of the

supernatant was added to 250 μl of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 500 ml of 1 M sodium acetate (pH 5.2) and then incubated at 50°C for 30 min. The amount of pyruvate produced was determined at OD_{335}.

The kinetic parameters (K_m and V_{max}) were computed from a Lineweaver–Burk transformation (V^{-1} vs. S^{-1}) of the Michaelis–Menten equation, where V is the formation rate of hydrogen sulfide (μmol/min/mg) and S is the concentration (M) of L-cysteine.

Preparation of Lcs mutants

pCDS1 containing the *lcs* gene was used for the preparation of Lcs mutants. Replacement of amino acids in the Lcs protein was carried out using a QuikChange site-directed mutagenesis kit (Stratagene) (22). The nucleotide sequences of the mutagenic PCR primers designed to replace a wild-type amino acid (Y59, Y118, D198, K233, or R365) with the other amino acids are as follows: Y59A, 5′-CACGGTGTCTTCGGCGC TGCCATTGTGGGCGAC-3′; Y118A, 5′-ATACAGACACCAGTCGCCAATTG TTTCTATTCT-3′; D198N, 5′-GTGAAG GTGATAAGCAACGAAATTCATTGCG AG-3′; K233N, 5′-TTCAATTCGCCTAC CAATAACTTCAACATCGCA-3′; R365G, 5′-GGCGAGGGCTATATCGGAATCAAC CTTGCTTGC-3′, and their complementary oligonucleotides primers, respectively. The mutated bases are underlined on each primer. The mutations generated in the *lcs* gene were confirmed by nucleotide sequencing. The resultant mutated *lcs* gene was expressed in *E. coli* BL21 cells and purified using the GST purification system as described above (23).

Assay of hemolysis and hemoglobin modification

Hemolysis assay was performed as described by Chu et al. with some modifications (7). Hemoglobin and modified-hemoglobin in sheep erythrocytes (RBCs) were measured as described previously (47) with some modifications. Briefly, RBCs were washed three times with phosphate-buffered saline to remove the soluble hemoglobin before the RBCs were used. The reaction mixture consisted of 120 mM NaCl, 10 mM Na_2HPO_4, 5 mM KH_2PO_4 (pH 7.5), 10 μM PLP, 4 μg/ml Lcs and 4% (volume/volume) RBCs with 1 mM L-cysteine. The mixture was incubated at 37°C with slow rotation and was withdrawn at 0, 3, 6, 9, and 12 h for measuring the hemolysis of RBCs and the hemoglobin and modified-hemoglobin in RBCs. Hemolysis was estimated by measuring the unbroken RBCs in the reaction mixture at OD_{405}. The percent hemolysis was calculated as described previously (7). The hemoglobin-modification assay was carried out as follows: the reaction mixture withdrawn at the time indicated above was centrifuged to collect the RBCs. The RBCs were washed several times, suspended in phosphate-buffered saline and then lysed by the addition of distilled water. Hemoglobin and modified-hemoglobin in the lysate were measured at OD_{540} and OD_{620}, respectively, to observe the changes in the amounts of hemoglobin and modified-hemoglobin in RBCs (47).

Nucleotide sequence accession number

The nucleotide sequence of *lcs* gene from *P. intermedia* ATCC25611 reported in this paper has been deposited in the DNA Data Bank of Japan nucleotide sequence databases with accession number AB481366.

Results

Analysis of L-cysteine desulfhydrase gene from *P. intermedia* ATCC25611

To determine and analyse the L-cysteine desulfhydrase gene from *P. intermedia* ATCC25611, a homologue of the gene was searched on the genome database of *P. intermedia* strain 17 on the basis of the amino acid sequences of Hly (6), Lcd (47), MalY (50), and PatB (1), which were

```
                                                                                                60
Lcs     MGKYNFDELVNRRGTNSVKWDEEKE------DGIIPLWVADMDFLAAPAIRRAVEERAKH
Lcd     MSKYNFQTAPNRLSHHTYKWKETET-----DPQLLPAWIADMDFEVMPEVKQAIHDYAEQ
Hly     -MIYDFTTKISRKNLGSLKWDLMYSQNPEVGNEVVPLSVADMEFKNPPELIEGLKKYLDE
MalY    --MFDFSKVVDRHGTWCTQWDYVADRFG--TADLLPFTISDMDFATAPCIIEALNQRLMH
PatB    ---MNFDKREERLGTQSVKWDKTGELFG--VTDALPMWVADMDFRAPEAITEALKERLDH
                *       *       *                 *     ** *

                                                                                               120
Lcs     GVFGYAIV-GDSYYAAITNWFKRRHNWAIERDWIIYTTGVIPAISATIHALAMPGEKVLI
Lcd     LVYGYTYA-SDELLQAVLDWEKSEHQYSFDKEDIVFVEGVVPAISIAIQAFTKEGEAVLI
Hly     TVLGYTGP-TEEYKKTVKKWMKDRHQWDIETDWIINTAGVVPAVFNAVREFTKPGDGVII
MalY    GVFGYSRWKNDEFLAAIAHWFSTQHYTAIDSQTVVYGPSVIYMVSELIRQWSETGEGVVI
PatB    GIFGYTTP-DQKTKDAVCGWMQNRHGWKVNPESITFSPGVVTALSMAVQAFTEPGDQVVV
          **              *     *                 *                 *   *

                                                                                               180
Lcs     QTPVYNCFYSCIRNQGLRVLESPLKREDDTYVVDWDDFEAKCADKKTTLFLLCTPHNPAG
Lcd     NSPVYPPFARSVRLNNRKLVSNSLKEENGLFQIDFEQLEKDIVENNVKLYLLCSPHNPGG
Hly     ITPVYYPFFMAIKNQERKIIECELLEKDGYYTIDFEKLEKLSKDKNNKALLFCSPHNPVG
MalY    HTPAYDAFYKAIEGNQRTVMPVALEKQADGWFCDMGKLEAVLAKPECKIMLLCSPQNPTG
PatB    QPPVYTPFYHMVEKNGRHILHNPLLEKDGAYAIDFEDLETKLSDPSVTLFILCNPHNPSG
          * *   *                 *           *     *                 * * ** •

                                                                                               240
Lcs     RVWSKDELARMNEICMRHDVKVISDEIHCELIMPGYVFTPFATVNADCQRNSVVFNSPTK
Lcd     RIWEREVLERIGHLCQKHQVILVSDEIHQDLTLFSHEHVSFNTISPDFKEFAIILSSATK
Hly     RVWKKDELQKIKDIVLKSDLMLWSDEIHFDLIMPGYEHTVFQSIDEQLADKTITFTAPSK
MalY    KVWTCDELEIMADLCERHGVRVISDEIHMDMVWGEQPHIPWSNVAR---GDWALLTSGSK
PatB    RSWSREDLLKLGELCLEHGVTVVSDEIHSDLMLYGHKHTPFASLSDDFADISVTCAAPSK
          *     *                 *• ***                             •

                                                                                               300
Lcs     NFNIAGLQIANIICADKEWYRRIDRAIN-IFEVCDVNPFGPLALEAAYNESEDWIDELMP
Lcd     TFNIAGTKNSYAVIENPSLRAQFQRQQL-ANNHHEVSSLGYIATETAYRYGKPWLVALKA
Hly     TFNIAGMGMSNIIIKNPDIRERFTKSRD-ITSGMPFTTLGYKACEICYKECGKWLDGCIK
MalY    SFNIPALTGAYGIIENSSSRDAYLSALKGRDGLSSPSVLALTAHIAAYQQGAPWLDALRI
PatB    TFNIAGLQASAIIIPDRLKRAKFSASLQ-RNGLGGLNAFAVTAIEAAYSKGGPWLDELIT
          *** *                                   *     *     *

                                                                                               360
Lcs     YIADNYALLKDTFAKEVPNYEVLKLEGTYLAWVDIRKSGLTANALTEKLLREGKVQVNSG
Lcd     VLEENIKFAVDYFAKEAPRLKVMKPQGTYLIWLDFSDYGLTDDELFTLLHDQAKVILNRG
Hly     VIDKNQRIVKDFFEVNHPEIKAPLIEGTYLQWIDFRALKMDHKAMEEFMIHKAQIFFDEG
MalY    YLKDNLTYIADKMNAAFPELNWQIPQSTYLAWLDLRPLNIDDNALQKALIEQEKVAIMPG
PatB    YIEKNMNEAEAFLSTELPKVKMMKPDASYLIWLDFSAYGLSDAELQQRMLKKGKVILEPG
          *               *           ** * *                         *


Lcs     VIYSKNDGEGYIRINLACPRATLQEGLKRIVNVLKACV-----
Lcd     SDYGKE-GELHARLNIATPKPLVEEICKRIVHCLPQ-------
Hly     YIFGDG-GIGFERINLAAPSSVIQESLERLNKALKDLKNRHLK
MalY    YTYGEE-GRGFVRLNAGCPRSKLEKGVAGLINAIRAVR-----
PatB    TKYGPG-GEGFMRLNAGCSLATLQDGLRRIKAALS--------
                *     •  *
```

Fig. 1. Alignment of Lcs with Lcd, MalY, Hly, and PatB. The four residues previously reported to be invariant in aminotransferases (34, 35) are marked by closed circles. The Lys_{233} (K_{233}) residue is the potential binding site of PLP. The eight residues that are found in most aminotransferases are boxed. Asterisks indicate amino acids that are identical in all five sequences. Lcs, Lcs protein from *Prevotella intermedia* (this study); Lcd, Lcd protein from *Streptococcus anginosus* (45); MalY, MalY protein from *Escherichia coli* (49); Hly, Hly protein from *Treponema denticola* (6); and PatB, PatB protein from *Bacillus subtilis* (1).

previously reported as L-cysteine desulfhydrase. The PI0305 gene on the genome of strain 17 was found as the homologue with high similarity. Based on the PI0305 gene sequence from strain 17, we determined the entire DNA sequence (1170 bp) of the PI0305 gene from *P. intermedia* ATCC 25611 and named this gene *lcs*. The *lcs* gene of strain ATCC25611 and the PI0305 gene from strain 17 shared 98% identity in the nucleotide sequence. The deduced amino acid sequence revealed that this *lcs* gene encoded a protein consisting of 390 amino acid residues with a calculated molecular mass of 44,381 Da and an isoelectric point of 5.61. Lcs showed 35% identity with Hly and Lcd, 39% with MalY, and 33% with PatB. As shown in Fig. 1, the aligned amino acid sequence of Lcs with Hly, Lcd, MalY, and PatB showed four invariant residues found in the α-family of aminotransferases, and one of them was the putative PLP-binding residue, Lys (K), at position 233 in the Lcs (34, 35). In addition, six of the eight residues that are found in most aminotransferases in the α-family are conserved.

Purification of Lcs from *P. intermedia* ATCC25611

To confirm that the nucleotide sequence determined in this study specifies the Lcs enzyme, pCDS1 containing the *lcs* gene homologue was expressed in *E. coli* BL21 and purified by the GST purification system. The recombinant Lcs was purified as a single band with the molecular weight of 44 kDa (Fig. 2A), which was consistent with the predicted molecular mass (44.4 kDa). The enzyme activity of the recombinant Lcs was confirmed by active staining on native-PAGE (see Assay II). As shown in Fig. 2C (lane 2), Lcs activity was detected as a single black band on the gel, confirming that the recombinant Lcs protein has the ability to produce hydrogen sulfide from L-cysteine. In addition, Lcs activity in crude extract from *P. intermedia* ATCC25611 was also detected as identical in size to the recombinant Lcs (Fig. 2C, lane 1) although Coomassie blue staining did not show an apparent Lcs protein band in the crude extract (Fig. 2B, lane 1).

Characterization of the *P. intermedia* Lcs

To characterize the purified recombinant Lcs enzyme, we examined substrate specificity, kinetic parameters (K_m and V_{max}), and optimum pH of the enzyme. The substrate specificity of the enzyme was examined by its ability to generate hydrogen sulfide from the SH-containing compounds such as L-cysteine, DL-homocysteine, cysteinyl glycine, and glutathione. The L-cysteine was a sole substrate for the *P. intermedia* Lcs enzyme, but DL-homocysteine, cysteinyl glycine, and glutathione were not substrates to produce hydrogen sulfide (data not shown). The K_m and V_{max} were determined for Lcs activity with L-cysteine as a substrate. The K_m and V_{max} were 0.7 mM and 4.2 μmol/min/mg of protein, respectively. The effect of pH on Lcs activity was tested by using 50 mM phosphate buffer at a pH range of 5.5–8.0. Maximum enzyme activity (hydrogen sulfide production) was observed at pH 7.0.

Essential amino acids for Lcs activity

The crystal structure of Hly from *T. denticola* proposed the active site structure and the catalytic mechanism for Hly (28), suggesting that a part of the amino acid residues in the proposed active site are closely related to the catalytic reaction mechanism. On the basis of this report, we focused on amino acid residues such as Tyr-64, Tyr-123, Asp-203, Lys-238, and Arg-369, which are responsible for the reaction mechanism of Hly, and searched for the corresponding residues on the Lcs sequence. Therefore, the five amino acid residues (Tyr-59, Tyr-118, Asp-198, Lys-233, Arg-365) in the Lcs molecule were targeted and converted to Ala, Ala, Asn, Asn, and Gly, respectively, by site-directed mutagenesis, generating Tyr-59-Ala (Y59A), Tyr-118-Ala (Y118A), Asp-198-Asn (D198N), Lys-233-Asn (K233N), and Arg-365-Gly (R365G) mutants. As shown in Fig. 3A, each mutant was successfully expressed in *E. coli* BL21 and purified as a homogeneous protein with a single band of 44 kDa on sodium dodecyl sulfate–PAGE.

As shown in active staining (Fig. 3B), Y59A, Y118A, D198N, and K233N failed to display enzyme activity, implying that Tyr-59, Tyr-118, Asp-198, and Lys-233 were essential for Lcs enzyme activity. Lcs activity was also confirmed by hydrogen sulfide production using Assay I. Y59A, Y118A, D198N, and K233N did not exhibit hydrogen sulfide production although only R365G retained catalytic activity as well as the wild-type enzyme (data not shown).

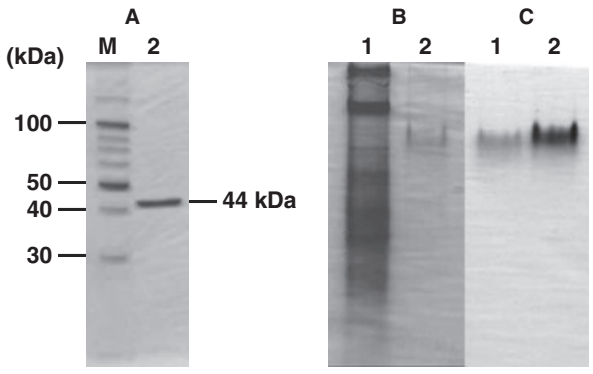

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) profile of L-cysteine desulfhydrase. (A) Sodium dodecyl sulfate–PAGE (12.5% acrylamide) of recombinant Lcs purified from *Escherichia coli* cells. The gel was stained with Coomassie brilliant blue. (B, C) Native PAGE (12.5% acrylamide) of Lcs enzyme. The sample was analysed by native PAGE and then the gel was stained with (B) Coomassie brilliant blue or (C) activity staining. Lanes 1, crude extract from *Prevotella intermedia* ATCC 25611; lanes 2, recombinant Lcs purified from *Escherichia coli* cells; M, size marker. Crude extract was prepared as follows: cell suspension of strain ATCC25611 was sonicated and centrifuged. The resultant supernatant was used as a crude extract.

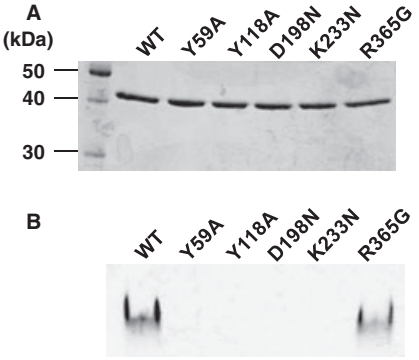

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) analysis of L-cysteine desulfhydrase mutants. (A) The mutant enzymes were subjected to sodium dodecyl sulfate–PAGE with 12.5% acrylamide and then stained with Coomassie brilliant blue. (B) The mutant enzymes were analysed by native PAGE with 12.5% acrylamide and then actively stained. Equal amounts of mutant proteins were added to each lane in (A) and (B).

Effects of metal ions on Lcs activity

As it is reported that the zinc ion inhibits the formation of volatile sulfur compounds (24, 48, 49), we examined the effects of divalent metal ions containing the zinc ion on Lcs activity. Effects of divalent metal ions on Lcs activity were examined by the production of hydrogen sulfide (Assay I) and pyruvate (Assay III). $CaCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ was added to the Lcs reaction mixture at a final concentration of 1 mM in each assay. Only $ZnCl_2$ strongly inhibited the production of hydrogen sulfide, but the other metal ions showed essentially no effect (data not shown). In addition, both pyruvate and hydrogen sulfide were measured under the presence of 0.01–1 mM $ZnCl_2$. Production of both reaction end-products decreased gradually when increased amounts of $ZnCl_2$ were added to the Lcs reaction mixture (Table 1). In contrast, the addition of $CaCl_2$, $MgCl_2$, or $MnCl_2$ did not influence pyruvate production (data not shown).

Hemolysis and hemoglobin-modification

It has been suggested that hydrogen sulfide modifies hemoglobin to primarily sulfhemoglobin and methemoglobin (29, 47), and the modified hemoglobin results in the lysis of RBCs (47). Therefore, we examined the effect of Lcs on hemolysis and hemoglobin-modification. As shown in Fig. 4A, RBCs lysed in the reaction mixture with both Lcs and L-cysteine although hemolysis was not observed with either Lcs or L-cysteine as a control. We also confirmed that incubation of RBCs with another reaction product such as pyruvate or ammonia did not produce hemolysis (data not shown). In this reaction, production of hydrogen sulfide reached a maximum at 30 min incubation (data not shown), which preceded hemolysis and hemoglobin-modification.

In addition, we measured the amounts of hemoglobin and modified-hemoglobin in the unbroken RBCs in the Lcs reaction mixture. As described in Materials and methods, lysate obtained by lysis of RBCs was used as the sample for determining hemoglobin (at OD_{540}) and modified-hemoglobin (at OD_{620}) (47). As shown in Fig. 4, the amount of hemoglobin in the unbroken RBCs decreased in a time-dependent manner for the initial 6-h incubation (Fig. 4B). In contrast, the amount of modified-hemoglobin increased in a time-dependent manner (Fig. 4C). These findings suggest that hemoglobin changed to modified-types of hemoglobin in RBCs.

Discussion

P. intermedia is thought to be closely associated with a variety of periodontal diseases and are able to produce various virulence factors that play an important role in the initiation and progression of periodontal diseases. Hemolytic activity contributes to the pathogenicity of *P. intermedia* (39, 43); however the mechanisms of hemolysis are unclear. In this study, we characterized the enzyme with hemolytic activity from *P. intermedia* ATCC25611. This enzyme catalysed L-cysteine to produce hydrogen sulfide, pyruvate, and ammonia, demonstrating that the enzyme is L-cysteine desulfhydrase. We named this enzyme Lcs. This type of enzyme has been genetically identified and characterized biochemically as Hly, Lcd, MalY, and PatB from *T. denticola* (9), *S. anginosus* (47), *E. coli* (50), and *Bacillus subtilis* (1), respectively. These reports reveal that these four enzymes belong to the α-family of PLP-dependent enzymes. As shown in Fig. 1, alignment data showed that Lcs retained four invariant residues found in the α-family of aminotransferases, and one (Lys) of which was the putative PLP-binding residue (34, 35). In addition, six of the eight residues that are found in most aminotransferases in the α-family were conserved. These findings indicate that Lcs also belongs to the α-family of aminotransferase and functions as a PLP-dependent cysteine desulfhydrase.

Substrate specificity of the Lcs enzyme revealed that only L-cycteine residue is a possible substrate. However, Lcs was inactive against the SH-containing di- and tri-peptides such as L-cysteinyl glycine and glutathione. It is known that periodontal pathogens use proteins for their nutrition and therefore produce a variety of proteolytic enzymes that are capable of degrading periodontal tissues, tissue fluid, and immune proteins (14). As a result of the proteolytic degradation, proteins are ultimately broken-down into a variety of peptides and amino acids in the confines of the periodontal environment. L-Cysteine is likely to be present in this environment. Therefore, we speculate that the *P. intermedia* Lcs enzyme uses a cysteine which is supplied as free amino acid released from peptides by proteolytic degradation of periodontal pathogens including *P. intermedia*.

The crystal structure of cystalysin (Hly) from *T. denticola* proposed the active site structure and the catalytic mechanism for Hly (28). The superposition of the active sites of Hly with MalY revealed similar active site architectures and the conserved active site residues. In addition, the detailed catalytic mechanism for Hly suggested that part of the amino acid residues in the active site are closely responsible for catabolism of the substrate (28). Based on this information, we focused on the amino acid residues shown in the molecular reaction mechanism of Hly and chose five residues, Tyr-64, Tyr-123, Asp-203, Lys-238, and Arg-369, in the active site of Hly. Of these five residues, Tyr-64, Tyr-123, and Lys-238 are also suggested as the active site key residues in catalysis of a PLP-dependent enzyme reaction by Hly, MalY, and cystathionine β-lyase (28). Therefore, we searched the corresponding amino acid residues on the Lcs sequence and found five residues: Tyr-59, Tyr-118, Asp-198, Lys-233, and Arg-365. Replacement of these amino acids by site-directed mutagenesis exhibited that Tyr-59, Tyr-118, Asp-198, and Lys-233 are essential for Lcs enzyme activity (Fig. 3). This is the first report that identified the amino acids that are indispensable for the activity of L-cysteine desulfhydrase using a mutation study. Further mutational analysis will be needed to clarify other amino acid residues responsible for Lcs enzyme activity.

Zinc ions inhibit the formation of VSC, and thereby reduce or inhibit oral malodor (24, 48, 49). It is thought that zinc ions form insoluble sulfides by interacting with the sulfur in precursors of VSC, so inhibiting further production of VSC. In the present study, production of hydrogen sulfide in a Lcs reaction mixture was completely inhibited by 1 mM $ZnCl_2$ (Table 1). In this case, at least two inhibition modes could be suggested: one is formation of insoluble sulfides by interacting zinc ions with hydrogen sulfide as described above, and the other is inhibition

Table 1. Effect of $ZnCl_2$ on recombinant L-cysteine desulfhydrase

	Production (μmol/min/mg)			
$ZnCl_2$	0 mM	0.01 mM	0.1 mM	1 mM
Pyruvate	5.26	4.76	2.53	< 0.01
Hydrogen sulfide	8.37	7.34	4.70	< 0.01

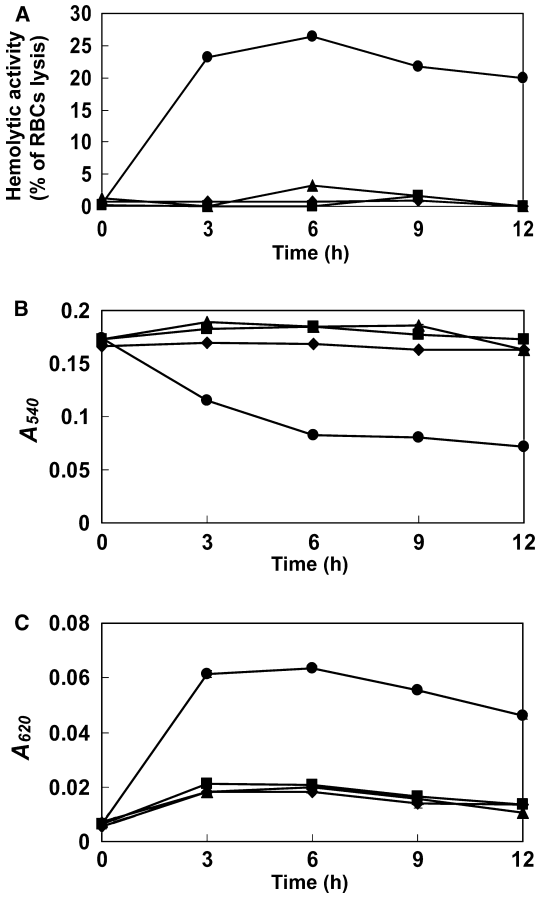

Fig. 4. Kinetics of hemolytic activity (A). Changes in the amounts of hemoglobin (B) and modified-hemoglobin (C) in unbroken red blood cells (RBCs). The reaction mixture containing L-cysteine, L-cysteine desulfhydrase, and RBCs was incubated at 37°C with slow rotation for the time indicated. After centrifugation, the pellets (unbroken RBCs) were broken by the addition of distilled water to release hemoglobin (B) and modified-hemoglobins (C) from unbroken RBCs. □, RBCs only; ■, RBCs plus 1 mM L-cysteine; ▲, RBCs plus L-cysteine desulfhydrase; ●, RBCs plus 1 mM L-cysteine plus L-cysteine desulfhydrase. Each graph is representative of three similar experiments.

of the Lcs enzyme by zinc ions. As shown in TABLE 1, production of not only hydrogen sulfide but also pyruvate gradually decreased in the presence of increased amounts of $ZnCl_2$. Neither end-product was detected under the presence of 1 mM $ZnCl_2$. This result strongly suggests that zinc ions interact with the Lcs enzyme (L-cysteine desulfhydrase), resulting in loss of Lcs enzyme activity. This also means that zinc ions have an excellent anti-hydrogen sulfide effect.

We suggested that the Lcs enzyme from *P. intermedia* is a cysteine-dependent enzyme that produces hydrogen sulfide, pyruvate, and ammonia as end-products and is responsible for hemoglobin-modification and hemolysis. Although these phenomena were observed only when RBCs were exposed to Lcs and L-cysteine (Fig. 4A,C), exposure of RBCs to either pyruvate or ammonia, or both, did not influence hemolysis and modification of hemoglobin (data not shown). The result revealed that hydrogen sulfide produced by Lcs is closely associated with hemoglobin-modification and hemolysis. These observations are supported by the following reports. Moxness et al. (37) provided a general mechanism to connect sulfhemoglobin formation to erythrocyte lysis. A series of Hly studies have shown that hemoglobin modification by hydrogen sulfide always precedes hemolysis and that the cysteine-dependent lysis of erythrocytes caused by Hly is the result of hydrogen sulfide production, resulting in the accumulation primarily of sulfhemoglobin and methemoglobin (6–10). Our present finding also showed that, in the Lcs reaction mixture, the amount of hydrogen sulfide produced by the Lcs enzyme reached a maximum at about 30-min of incubation (data not shown); subsequently, lysis of RBCs and modification of hemoglobin were observed (Fig. 4). These results suggest that hydrogen sulfide modifies hemoglobin and the formation and accumulation of modified-hemoglobin seem to play an important role in cysteine-dependent hemolysis as proposed by Hly studies (6–10).

Iron and heme are essential nutrients for periodontal pathogens and play a crucial role in their pathogenesis. Within the human host the majority of iron is found intracellularly in the form of hemoglobin, heme proteins, or ferritin (16) and small quantities of extracellular iron are complexed to iron-binding proteins, primarily transferrin and lactoferrin. Therefore, under physiological conditions, free hemoglobin or hemin may not be available because these compounds are efficiently scavenged by serum proteins haptoglobin and hemopexin, respectively (13, 16, 38). To survive in the iron-limited environment of the host, periodontal pathogens have multiple iron acquisition systems. *P. intermedia*, the etiological agent of periodontal diseases, also requires iron for growth (18), but lacks the ability to synthesize heme, an important growth factor for the bacterium. *P. intermedia* has the ability to bind to hemoglobin and utilize hemoglobin as an effective heme source (18, 31, 32) although this bacterium cannot utilize inorganic iron and iron-binding proteins such as transferrin and lactoferrin for growth, indicating that hemoglobin appears to be a more effective iron source (19, 30). To acquire hemoglobin, *P. intermedia* has hemolytic and hemagglutinating activities (4, 39, 42). A hemolytic substance (hemolysin) activated by reductants has been partially purified (43) and the gene encoding multiple hemolytic domains has been cloned in *P. intermedia* (3), but their mechanisms for hemolysis are still unclear because hemolytic substances have not been identified and characterized. As compared with our Lcs results, the property of the hemolytic substance and the nucleotide sequence encoding multiple hemolytic domains were distinct from those of our Lcs enzyme. These observations indicate that *P. intermedia* has several mechanisms for hemolysis, one of which is cysteine-dependent hemolysis by the Lcs enzyme as presented here. Our findings also suggest that inhibition of the Lcs enzyme by $ZnCl_2$ is effective for the prevention of oral malodor and periodontal diseases.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C)

(No. 18592009) and by a Grant-in-Aid for the Young Scientists (B) (No. 19791350) from the Ministry of Education, Sciences, Sports, and Culture of Japan.

References

1. Auger S, Gomez MP, Danchin A, Martin-Verstraete I. The PatB protein of *Bacillus subtilis* is a C-S-lyase. Biochimie 2005: **87**: 231–238.
2. Beauchamp RO Jr, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA. A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol 1984: **13**: 25–97.
3. Beem JE, Nesbitt WE, Leung KP. Cloning of *Prevotella intermedia* loci demonstrating multiple hemolytic domains. Oral Microbiol Immunol 1999: **14**: 143–152.
4. Been JE, Nesbitt WE, Leung KP. Identification of hemolytic activity in *Prevotella intermedia*. Oral Microbiol Immunol 1998: **13**: 97–105.
5. Carlsson J, Larsen JT, Edlund MB. *Peptostreptococcus micros* has a uniquely high capacity to form hydrogen sulfide from glutathione. Oral Microbiol Immunol 1993: **8**: 42–45.
6. Chu L, Burgum A, Kolodrubetz D, Holt SC. The 46-kilodalton-hemolysin gene from *Treponema denticola* encodes a novel hemolysin homologous to aminotransferases. Infect Immun 1995: **63**: 4448–4455.
7. Chu L, Ebersole JL, Holt SC. Hemoxidation and binding of the 46-kDa cystalysin of *Treponema denticola* leads to a cysteine-dependent hemolysis of human erythrocytes. Oral Microbiol Immunol 1999: **14**: 293–303.
8. Chu L, Ebersole JL, Kurzban GP, Holt SC. Cystalysin, a 46-kilodalton L-cysteine desulfhydrase from *Treponema denticola*: biochemical and biophysical characterization. Clin Infect Dis 1999: **28**: 442–450.
9. Chu L, Ebersole JL, Kurzban GP, Holt SC. Cystalysin, a 46-kilodalton cysteine desulfhydrase from *Treponema denticola*, with hemolytic and hemoxidative activities. Infect Immun 1997: **65**: 3231–3238.
10. Chu L, Holt SC. Purification and characterization of a 46-kDa hemolysin from *Treponema denticola* ATCC 35404. Microb Pathog 1994: **16**: 197–212.
11. Claesson R, Edlund MB, Persson S, Carlsson J. Production of volatile sulfur compounds by various *Fusobacterium* species. Oral Microbiol Immunol 1990: **5**: 137–142.
12. Coli JM, Tonzetich J. Characterization of volatile sulphur compounds production at individual gingival crevicular sites in humans. J Clin Dent 1992: **3**: 97–103.
13. Dobyszycka W. Biological functions of haptoglobin – new pieces to an old puzzle. Eur J Clin Chem Clin Biochem 1997: **35**: 647–654.
14. Eley BM, Cox SW. Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects in host defenses and tissues and detection in gingival crevice fluid. Periolontol 2000 2003: **31**: 105–124.
15. Fukamachi H, Nakano Y, Yoshimura M, Koga T. Cloning and characterization of the L-cysteine desulfhydrase gene of *Fusobacterium nucleatum*. FEMS Microbiol Lett 2002: **215**: 75–80.
16. Genco CA, Dixon DW. Emerging strategies in microbial heme capture. Mol Microbiol 2001: **39**: 1–11.
17. Gharbia SE, Haapasalo M, Shah HN et al. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. J Periodontol 1994: **65**: 56–61.
18. Gibbons RJ, Macdonald JB. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J Bacteriol 1960: **80**: 164–170.
19. Guan S-M, Nagata H, Shizukuishi S, Wu J-Z. Degradation of human hemoglobin by *Prevotella intermedia*. Anaerobe 2006: **12**: 279–282.
20. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994: **5**: 78–111.
21. Holt SC, Bramanti TE. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit Rev Oral Biol Med 1991: **5**: 150–155.
22. Igarashi T, Morisaki H, Yamamoto Y, Goto N. An essential amino acid residue for catalytic activity of the dextranase of *Streptococcus mutans*. Oral Microbiol Immunol 2002: **17**: 193–196.
23. Igarashi T, Yamamoto Y, Goto N. Nucleotide sequence and molecular characterization of a dextranase gene from *Streptococcus downei*. Microbiol Immunol 2001: **45**: 341–348.
24. Jonski G, Young A, Waler SM, Rolla G. Insoluble zinc, cupric, and tin pyrophosphates inhibit the formation of volatile sulphur compounds. Eur J Oral Sci 2004: **112**: 429–432.
25. Kleinberg I, Westbay G. Oral malodor. Crit Rev Oral Biol Med 1990: **4**: 247–259.
26. Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. J Periodontal Res 1980: **15**: 111–122.
27. Krespi YP, Shrime MG, Kacker A. The relationship between oral malodor and volatile sulfur compound-producing bacteria. Otolaryngol Head Neck Surg 2006: **135**: 671–676.
28. Krupka HI, Huber R, Holt SC, Clausen T. Crystal structure of cystalysin from *Treponema denticola*: a pyridoxal 5′-phosphate-dependent protein acting as a haemolytic enzyme. EMBO J 2000: **19**: 3168–3178.
29. Kurzban GP, Chu L, Ebersole JL, Holt SC. Sulfhemoglobin formation in human erythrocytes by cystalysin, an L-cysteine desulfhydrase from *Treponema denticola*. Oral Microbiol Immunol 1999: **14**: 153–164.
30. Leung KP, Folk SP. Effects of porphyrins and inorganic iron on the growth of *Prevotella intermedia*. FEMS Microbiol Lett 2002: **209**: 15–21.
31. Leung K, Nesbitt WE, Okamoto M, Fukushima H. Identification of a fimbriae-associated haemagglutinin from *Prevotella intermedia*. Microb Pathog 1999: **26**: 139–148.
32. Leung KP, Subramaniam PS, Okamoto M, Fukushima H, Lai CH. The binding and utilization of hemoglobin by *Prevotella intermedia*. FEMS Microbiol Lett 1998: **162**: 227–233.
33. Loesche WJ, Syed SA, Laughon BE, Stoll J. The bacteriology of acute necrotizing ulcerative gingivitis. J Periodontol 1982: **53**: 223–230.
34. Mehta PK, Christen P. Homology of pyridoxal-5′-phosphate-dependent aminotransferases with the *cob*C (cobalamin synthesis), *nif*S (nitrogen fication), *pab*C (p-aminobenzoate synthesis) and *mal*Y (abolishing endogenous induction of the maltose system) gene products. Eur J Biochem 1993: **211**: 373–376.
35. Mehta PK, Hale TI, Christen P. Evolutionary relationships among aminotransferases. Tyrosin aminotransferase, histidinol-phosphate aminotransferase, and aspartate aminotransferase are homologous proteins. Eur J Biochem 1989: **186**: 249–253.
36. Miyazaki H, Sakao S, Katoh Y, Takehara T. Correlation between volatile sulphur compounds and certain oral health measurements in the general population. J Periodontol 1995: **66**: 679–684.
37. Moxness MS, Brunauer LS, Huestis WH. Hemoglobin oxidation products extract phospholipids from the membrane of human erythrocytes. Biochemistry 1996: **35**: 7181–7187.
38. Muller-Eberhard U. Hemopexin. Methods Enzymol 1988: **163**: 536–541.
39. Okamoto M, Maeda N, Kondo K, Leung KP. Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1999: **178**: 299–304.
40. Persson S, Edlund MB, Claesson R, Carlsson J. The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microbiol Immunol 1990: **5**: 195–201.
41. Slots J, Listgarten MA. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. J Clin Periodontol 1988: **15**: 85–93.
42. Stubbs S, Lewis MA, Waddington RJ, Embery G. Hydrolytic and depolymerising enzyme activity of *Prevotella intermedia*. Oral Dis 1996: **2**: 272–278.
43. Takada K, Fukatsu A, Otake S, Hirasawa M. Isolation and characterization of hemolysin activated by reductant from *Prevotella intermedia*. FEMS Immunol Med Microbiol 2003: **35**: 43–47.
44. Tyrrell KL, Citron DM, Warren YA, Nachnani S, Goldstein EJ. Anaerobic bacteria cultured from the tongue dorsum of subjects with oral malodor. Anaerobe 2003: **9**: 243–246.
45. Van der Weijden GA, Timmerman MF, Reijerse E, Wolffe GN, Van Winkelhoff AJ, Van der Velden U. The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in selected subjects with periodontitis. J Clin Periodontol 1994: **21**: 583–588.
46. Yaegaki K, Sanada K. Biochemical and clinical factors influencing oral malodor in periodontal patients. J Periodontol 1992: **63**: 783–789.
47. Yoshida Y, Nakano Y, Amano A et al. *lcd* from *Streptococcus anginosus* encodes a C-S lyase with α,β-elimination activity that

degrades L-cysteine. Microbiology 2002: **148**: 3961–3970.
48. Young A, Jonski G, Rolla G. The oral anti-volatile sulphur compound effects of zinc salts and their stability constants. Eur J Oral Sci 2002: **110**: 31–34.
49. Young A, Jonski G, Rolla G. Inhibition of orally produced volatile sulfur compounds by zinc, chlorhexidine or cetylpyridinium chloride – effect of concentration. Eur J Oral Sci 2003: **111**: 400–404.
50. Zdych E, Peist R, Reidl J, Boos W. MalY of *Escherichia coli* is an enzyme with the activity of a beta C-S lyase (cystathionase). J Bacteriol 1995: **177**: 5035–5039.