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High production of methyl mercaptan by L-methionine-α-deamino-γ-mercaptomethane lyase from *Treponema denticola*

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Abstract

Methyl mercaptan is derived from L-methionine by the action of L-methionine-α-deamino-γ-mercaptomethane lyase (METase) and is a major component of oral malodor. This compound is highly toxic and is thought to play an important role in periodontal disease. We found that *Treponema denticola*, a member of the subgingival biofilm at periodontal disease sites, produced a large amount of methyl mercaptan even at low concentration of L-methionine. METase activity in a cell-free extract from *T. denticola* was detected by two-dimensional electrophoresis under non-denaturing conditions, and the protein spot that exhibited high METase activity was identified using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. The identified gene produced a METase with a K_m value for L-methionine (0.55 mM) that is much lower than those of METases previously identified in the other organisms. This result suggests that *T. denticola* is an important producer of methyl mercaptan in the subgingival biofilm.

Keywords: Methyl mercaptan; *Treponema denticola*; L-Methionine-α-deamino-γ-mercaptomethane lyase

Oral malodor arises mainly from the production of volatile sulfur compounds (VSCs) resulting from the microbial degradation of sulfur-containing amino acids, such as cysteine and methionine. The predominant VSCs in the breath are hydrogen sulfide and methyl mercaptan. Both compounds are highly toxic, with methyl mercaptan being highly so [1]. VSCs can increase the permeability of the oral mucosa [2] and decrease protein or collagen synthesis [3,4]. It is possible that the presence of methyl mercaptan within a periodontal pocket is involved in the induction or progression of periodontal disease.

Methyl mercaptan is produced from L-methionine by the enzymatic action of L-methionine-α-deamino-γ-mercaptomethane lyase (METase), which catalyzes the α,γ-elimination of L-methionine to produce α-ketobutyrate, methyl mercaptan, and ammonia. This enzyme has been detected in anaerobic, oral microorganisms, such as *Porphyromonas gingivalis* [5], *Fusobacterium nucleatum* [6], and *Treponema denticola*. Yoshimura et al. reported that the *mgl* gene encoding METase is associated with the pathogenicity of *P. gingivalis*, and the parent W83 strain and the *mgl*-deficient mutant M1217 were tested for virulence in a mouse model [24].

Treponema denticola is a small oral spirochete which is found in progressing periodontal lesions and is associated with periodontal disease [7,8]. This oral bacterium is reported as an active producer of methyl mercaptan

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from heat-inactivated serum, but *P. gingivalis* and *Porphyromonas endodontalis* produced about 10 times larger amount of methyl mercaptan [9]. On the other hand, *T. denticola*, as well as *P. gingivalis*, *P. endodontalis*, *Prevotella intermeria*, and *Prevotella loescheii*, was the most active species that produced hydrogen sulfide from heat-inactivated serum [9]. Several researchers have focused on this organism as a high producer of hydrogen sulfide, purified cysteine desulfhydrase or cystalysin, the cloning of the genes responsible [10], and determined the crystal structure of the enzyme from *T. denticola* [11].

In this study, we cloned the gene encoding METase from *T. denticola* and examined the kinetic properties of the gene product. Our results indicate that, among oral bacteria, *T. denticola* is an important producer of both hydrogen sulfide and methyl mercaptan.

Materials and methods

Bacterial strains and culture conditions. Treponema denticola ATCC 35405 was cultured anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37 °C in GM-1 medium [12]. The bacteria were inoculated at a ratio of 1/10 into the medium. After 2 days of growth, the optical density at 660 nm (OD_{660}) was approximately 0.26, and the culture was used as the inoculum for subsequent culture. *P. gingivalis* W83 was grown anaerobically in GAM broth (Nissui Medical, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). *Escherichia coli* BL21 was cultured aerobically at 37 °C in 2× TY broth.

Non-denaturing two-dimensional electrophoresis. The cellular proteins of *T. denticola* were evaluated by non-denaturing two-dimensional electrophoresis (2-DE) using a Multiphor II IEF system (Amersham Biosciences, Piscataway, NJ). Immobiline DryStrips (IPG strips, Amersham) pH 4–7 were rehydrated for 16 h in 4% 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (Chaps, Amersham Biosciences), 1 mM EDTA, 40 mM DL-dithiothreitol (ICN Biomedicals, St. Laurent, Quebec, Canada), 2% IPG buffer (Amersham Biosciences), 10% glycerol, and bromophenol blue. The bacterial pellet was suspended in lysis solution containing 4% Chaps, 40 mM Tris, 1 mM EDTA, and 2% IPG buffer. Cells were lysed by ultrasonication on ice and centrifuged for 15 min at 10,000*g*. Each protein sample (100 μg) was loaded onto an 11 cm IPG strip using a sample cup. First-dimensional separation by isoelectric focusing was initiated at 200 V; the voltage was gradually increased to 3500 V for 3000 Vh and remained at 3500 V for 35 kVh at 15 °C. After isoelectric focusing, proteins were separated in the second dimension by molecular mass using polyacrylamide gel electrophoresis under native conditions (native-PAGE) at a constant voltage of 200 V at 15 °C. Native-PAGE was performed as follows. The stacking gel contained 4% acrylamide, and the resolving gel contained 7.5% polyacrylamide with 1% Chaps and 10% glycerol. There was no sodium dodecyl sulfate in the gels or in the electrophoresis buffer.

Enzyme assay. METase catalyzes not only the α,γ-elimination of L-methionine to produce methyl mercaptan, α-ketobutyrate, and ammonia, but also catalyzes the α,γ-elimination of L-homocysteine to produce hydrogen sulfide, α-ketobutyrate, and ammonia [13–16]. Bismuth reacts with hydrogen sulfide forming a black precipitate, but not with methyl mercaptan, to form a black precipitate; thus, METase activity was detected in the polyacrylamide gel by using the method of Claesson et al. [17] to visualize the METase activity in polyacrylamide gel. In this method, after electrophoresis of the protein, the gel is soaked at 37 °C in a solution containing 100 mM triethanolamine–HCl (pH 7.6), 10 μM pyridoxal 5′-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100, and 20 mM homocysteine.

The kinetics of METase activity were assayed as described by Yoshimura et al. [5]. The kinetic parameters (K_m and V_{max}) were computed from the Hanes–Woolf plot (SV^{-1} versus S) of Michaelis–Menten equation, where V is the formation rate of α-ketobutyrate (μmol/min/mg) and S is the concentration (M) of L-methionine. The assay was carried out with 1 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxal 5′-phosphate and L-methionine. The enzyme was added to start the reaction. After incubation for 15 min at 37 °C, the reaction was terminated by adding 0.5 ml of 4.5% trichloroacetic acid. The suspension was centrifuged, and 0.5 ml of the supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate (pH 5.2) and then incubated at 50 °C for 30 min. The amount of α-ketobutyrate produced was determined using a spectrophotometer at a wavelength of 335 nm. The amount of protein was determined by using the Bio-Rad protein assay with bovine serum albumin as a standard.

Gas chromatography of VSCs produced by intact cells. Methyl mercaptan produced by intact bacterial cells was detected using a modification of the method of Yoshimura et al. [5]. *P. gingivalis* was grown at 37 °C until an optical density at 550 snm (OD_{550}) of about 0.6 was attained. The cells were harvested and washed with a buffered salt solution of 40 mM potassium phosphate buffer (pH 7.7) and 50 mM sodium chloride. The cells were resuspended in the salt solution to an OD_{550} of 0.3. *T. denticola* was grown at 37 °C to an OD_{550} of about 0.4. The cells were harvested, washed with buffered salt solution, and resuspended in the salt solution an OD_{550} of 0.3. A 970 μl volume of cell suspension diluted with the buffered salt solution to appropriate concentrations was placed in sterile tube and sealed with a silicon plug. The reaction was initiated by adding 30 μl of L-methionine. After incubation at 37 °C for 30 min, the reaction was stopped by adding 500 μl of 3 M phosphoric acid. A 1 ml sample of the vapor above the reaction mixture was removed with a gas-tight syringe and analyzed by gas chromatography (model GC-14B; Shimadzu Works, Tokyo, Japan) using a glass column packed with 25% β,β 9-oxydipropionitrile on a 60–80 mesh Chromosorb W AW-DMCS-ST device (Shimadzu Works) fitted with a flame photometric detector at 70 °C. The concentration of each VSC was determined based on a standard of hydrogen sulfide, methyl mercaptan, or dimethyl sulfide gas prepared with Parmeater PD-1B (GL Science, Tokyo, Japan). Methyl mercaptan production activity was expressed relative to the amount of protein in bacterial cell lysates as measured by the Lowry method.

Peptide mass fingerprinting. The protein spot showing METase activity was semi-automatically extracted, trypsinized, and purified using a ProteomIQ Xcise In-Gel Digest kit (Proteome Systems, MA) and a high throughput gel-excision processor (Xcise, Shimadzu Works). Peptide mass analysis was performed using an AXIMA CFR (Shimadzu Works) matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer in reflectron mode.

Preparation of an open reading frames database and protein identification. Database searching following peptide mass fingerprinting (PMF) made use of the MS-Fit software package containing the *T. denticola* ATCC 35405 genome, the sequence of which is available in GenBank as GI number 42516522. The genome sequence was translated using the GeneMark.hmm program (http://opal.biology.gatech.edu/GeneMark/hmmchoice.html), and 2786 ORFs were predicted. Parameters for protein identification included a mass tolerance of 150 ppm and a maximum of one missed cleavage per peptide. Peptide masses were assumed to be monoisotopic, and cysteines were assumed to be carbamidomethylated. All translated ORFs that matched PMF data were used to query the NCBI databases (http://www.ncbi.nlm.nih.gov/blast/) using the PSI-BLAST program.

Purification of T. denticola METase from the cloned gene. To obtain the product of the cloned *megL* gene from *T. denticola*, *E. coli* BL21 cells were transformed with a plasmid containing a fragment amplified by PCR using with *Taq* DNA polymerase (TaKaRa Bio., Tokyo, Japan) using the primers 5′-AAAAGGATCCAATAGAAAAGAAT TGGAAAAGTTG-3′ (sense) and 5′-AAAACCCGGGTTATTTAA

CAAGTTTTATCCAAGGC-3′ (antisense) which were designed from the *T. denticola megL* gene sequence. Both primers were designed to create *Bam*HI and *Sma*I restriction sites (underlined). The PCR product was double-digested with *Bam*HI and *Sma*I, and ligated to the *Bam*HI–*Sma*I double-digested pGEX-6P-1 expression vector (Amersham Biosciences) to produce pMGL11. The nucleotide sequence of the insert was determined to confirm that no mutations had been introduced. The transformant was grown in 2× TY broth with ampicillin (50 μg/ml) at 37 °C until an OD_{550} of 0.7 was attained. Isopropyl-β-thiogalactopyranoside was added to the culture to a final concentration of 1 mM, and the culture was grown for 4 h. The cells were harvested by centrifugation and lysed by ultrasonication. The cell extract was obtained by centrifugation. Binding to glutathione–Sepharose 4B medium (Amersham Biosciences), cleavage of the fusion protein by PreScission protease, and elution of the product were performed according to the manufacturers' instructions.

Results

Separation of T. denticola proteins by non-denaturing two-dimensional electrophoresis and detection of METase activity

Fig. 1 shows the non-denaturing 2-DE pattern of *T. denticola* proteins. In the pH range 4.0–7.0, approximately 280 protein spots were counted in a representative protein profile of *T. denticola* cells. This pattern was quite different from the denaturing 2-DE pattern, and the proteins separated under non-denaturing conditions were expected to retain functions such as enzyme activity. After the proteins from *T. denticola* were separated using non-denaturing 2-DE, an in-gel adaptation of the METase active staining assay was used to detect enzyme activity. One major protein spot was found to precipitate bismuth.

Identification of the amino acid sequence using TOF-MS

The protein that precipitated bismuth was cut from gel, and the gel slice was equilibrated for two periods of 15 min in equilibration buffer containing 6 M urea, 50 mM Tris–HCl (pH 8.8), 2% SDS, and 30% glycerol. For the first equilibration step, 1%(w/v) DL-dithiothreitol was added to reduce the protein. Thereafter the protein was carbamidomethylated with 4%(w/v) iodoacetamide and digested in-gel with trypsin. The resulting peptides were identified by peptide mass fingerprinting using MALDI-TOF-MS. These peptides matched with a single ORF in the *T. denticola* ATCC 35405 genome and thus provided only one possibility for protein identification. The ORF was designated *megL* because the gene product was the major component corresponding to METase activity in *T. denticala*.

Characterization of METase of T. denticola

To evaluate the enzymatic activity of *T. denticola* METase, the enzyme was purified by using an expression vector containing the *megL* gene of *T. denticola*. The enzyme migrated at approximately 43.5 kDa on SDS–PAGE gels (Fig. 2) and the single protein band that stained with CBB corresponded to the bismuth precipitate band. The K_m and V_{max} of the purified enzyme were 0.55 mM and 36.6 μmol/min/mg protein, respectively.

Formation of methyl mercaptan by intact cells of P. gingivalis and T. denticola

Porphyromonas gingivalis and *T. denticola* whole cells were tested for their ability to form methyl mercaptan from L-methionine (Fig. 3). The production of methyl mercaptan was expressed as an amount per milligram of bacterial cell protein content, rather than relative to OD_{550}, because the sizes of *P. gingivalis* and *T. denticola* cells are different. A larger amount of methyl mercaptan

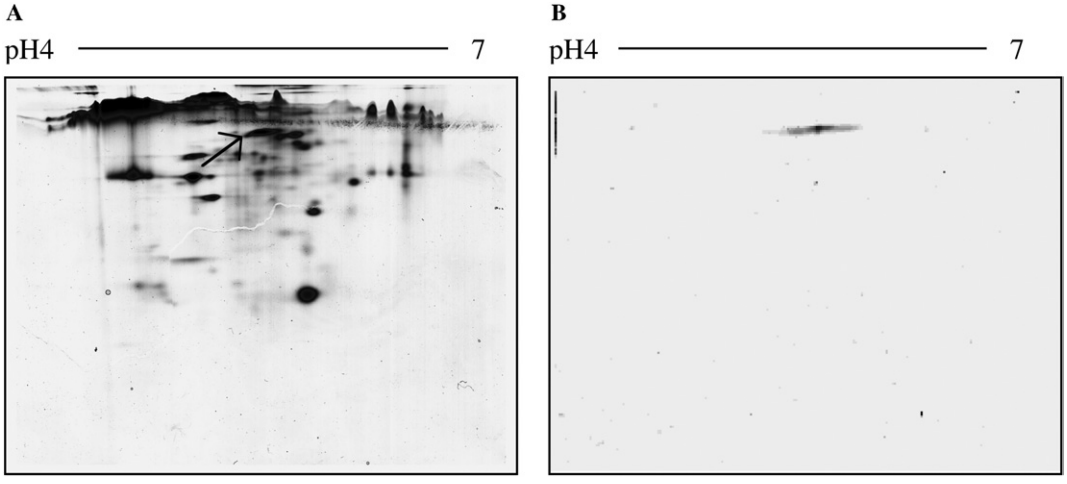

Fig. 1. Non-denaturing 2-DE patterns of *T. denticola* proteins. (A) Gel was stained with Coomassie brilliant blue R-250. (B) Gel was incubated at 37 °C in a solution containing 100 mM triethanolamine–HCl (pH 7.6), 10 μM pyridoxal 5′-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100, and 20 mM homocysteine in order to visualize the homocysteine desulfhydrase activity. A black precipitate was formed at the site of the METase enzyme in the gel.

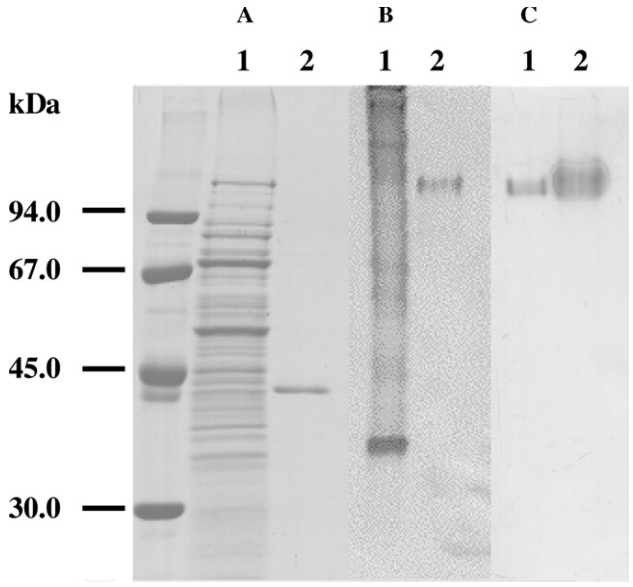

Fig. 2. PAGE analysis of METase activity on a 10% acrylamide gel. (A) The sample was analyzed by SDS–PAGE and stained with Coomassie brilliant blue R-250. (B) The sample was analyzed by native-PAGE, and the gel was stained with Coomassie brilliant blue R-250. (C) The sample was analyzed by native-PAGE, and homocysteine desulfhydrase activity was visualized as described in Fig. 1B. Lanes: 1, *T. denticola* ATCC 35405 crude extract; 2, purified recombinant METase.

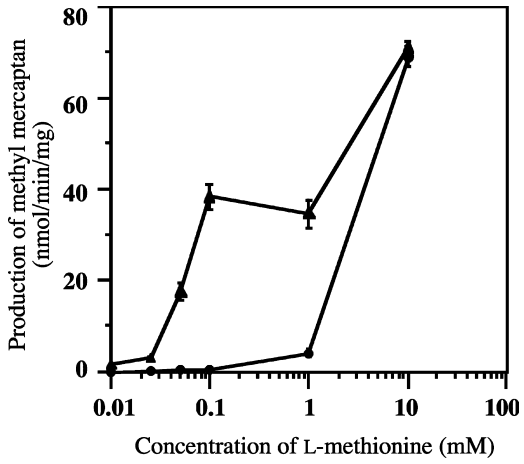

Fig. 3. The production of methyl mercaptan by intact cells. Closed triangles are *T. denticola*; closed circles are *P. gingivalis*. The values are expressed as means ± SD of triplicate assays.

was produced by *T. denticola* than by *P. gingivalis* at low substrate concentrations of 1 mM or less, whereas, at the high substrate concentration (10 mM), equivalent amounts of methyl mercaptan were produced by *P. gingivalis* (69.09 ± 2.15 nmol/min/mg) and *T. denticola* (71.14 ± 1.42 nmol/min/mg). *T. denticola* produced eight times more methyl mercaptan (34.56 ± 3.04 nmol/min/mg) than did *P. gingivalis* (4.09 ± 0.96 nmol/min/mg) in the presence of 1 mM methionine, and *T. denticola* produced about 95 times more methyl mercaptan (38.43 ± 2.81 nmol/min/mg) than did *P. gingivalis* (0.40 ± 0.09 nmol/min/mg) in the presence of 0.1 mM methionine.

Discussion

In recent years, there has been renewed interest in the correlation between oral malodor and periodontal disease [18]. Methyl mercaptan produced from L-methionine by the enzymatic action of METase is considered to be one of the main causes of oral malodor associated with periodontitis. Yaegaki and Sanada [19] reported that the ratio of methyl mercaptan to hydrogen sulfide was greater in the breath of patients with periodontal disease than in that of control subjects. Coli and Tonzetich [20] found a significantly higher methyl mercaptan/hydrogen sulfide ratio in deep or inflamed crevicular sites than in shallow or non-inflamed site. These findings suggest that methyl mercaptan from periodontal pockets may be associated with the oral malodor of patients with periodontitis. In addition, Yoshimura et al. [5] reported that there was a significant difference in the survival rate of mice infected with *P. gingivalis* W83 and strain M1217, the *megL*-deficient mutant, four days after injection and concluded that methyl mercaptan plays a significant role in pathogenicity. Understanding the mechanism of methyl mercaptan production in periodontal pockets may help in elucidating the source of oral malodor and in understanding the pathology of periodontal disease. In order to do this, we have studied the metabolic pathways that form VSCs in periodontal bacteria.

In this study, we detected METase activity in *T. denticola* using non-denaturing 2-DE and by examining the degradation of L-homocysteine. The separation of proteins using 2-DE under non-denaturing conditions allows the investigation of the functional aspects of the proteome, such as enzymatic activity, that are lost during denaturing 2-DE. This method is useful for screening enzyme activity, which can be exhibited by active staining in the gel. When the substrate was changed from L-homocysteine to L-cysteine, cystalysin, a major producer of hydrogen sulfide [10], was identified (data not shown). This enzyme catalyzes an α,β-elimination reaction with L-cysteine, yielding hydrogen sulfide, pyruvate, and ammonia, but does not catalyze an α,γ-elimination reaction. Both C-S lyases could be distinguished from one another with this method.

The purified *T. denticola* METase had a high affinity for L-methionine. The K_m values of the enzymes from *P. gingivalis*, *Clostridium sporogenes*, *Pseudomonas putida*, and *Trichomonas vaginalis* for L-methionine have been reported as 23.1 mM [6], 90 mM [15], 1 mM [21], and 4.3 mM [22], respectively, while that of the enzyme from *T. denticola* was 0.55 mM. This high affinity of the *T. denticola* METase for L-methionine suggests that

T. denticola has the ability to produce methyl mercaptan from low concentrations of L-methionine in the oral environment, where it is likely that there is no more than 20 mM free methionine present. It has been reported that 3.7 μM methionine is present in stimulated saliva [23]. In addition, about 26 μM methionine is present in serum [24], and so it would be expected that similar levels of methionine would be present in the gingival fluid or tissue fluids derived from serum. When the substrate was added at the high concentration, equivalent amounts of methyl mercaptan were produced by *P. gingivalis* and *T. denticola* cells. *T. denticola* produced about eight times more methyl mercaptan than did *P. gingivalis* in the presence of 1 mM methionine and about 95 times more in the presence of 0.1 mM methionine. This indicates that *T. denticola* is capable of producing high amounts of methyl mercaptan at low substrate concentration. *T. denticola* may be a more important producer of methyl mercaptan in the oral cavity than *P. gingivalis*.

Methyl mercaptan and other volatile sulfur compounds are thought to be highly toxic. For this reason, METase from *T. denticola* may play a role in the destruction of host cells through the production of methyl mercaptan. Methyl mercaptan may not only cause oral malodor but also play an important role in the pathogenicity of *T. denticola*. As METase has not been found in mammals, the inhibition of this enzyme should have little effect on humans. Therefore, we hypothesize that this enzyme may potentially be an exploitable target for novel chemotherapeutic drugs. An inhibitor of *T. denticola* METase may represent a new class of compounds with the potential for preventing and treating oral malodor.

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References

[1] C. Scully, M. el-Maaytah, S.R. Porter, J. Greenman, Breath odor: etiopathogenesis, assessment and management, Eur. J. Oral Sci. 105 (1997) 287–293.
[2] W. Ng, J. Tonzetich, Effect of hydrogen sulfide and methyl mercaptan on the permeability of oral mucosa, J. Dent. Res. 63 (1984) 994–997.
[3] P. Johnson, K. Yaegaki, J. Tonzetich, Effect of methyl mercaptan on synthesis and degradation of collagen, J. Periodontal Res. 31 (1996) 323–329.
[4] P.W. Johnson, K. Yaegaki, J. Tonzetich, Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts, J. Periodontal Res. 27 (1992) 553–561.
[5] M. Yoshimura, Y. Nakano, Y. Yamashita, T. Oho, T. Saito, T. Koga, Formation of methyl mercaptan from L-methionine by *Porphyromonas gingivalis*, Infect. Immun. 68 (2000) 6912–6916.
[6] M. Yoshimura, Y. Nakano, H. Fukamachi, T. Koga, 3-Chloro-DL-alanine resistance by L-methionine-α-deamino-γ-mercaptomethane-lyase activity, FEBS Lett. 523 (2002) 119–122.
[7] S.C. Holt, T.E. Bramanti, Factors in virulence expression and their role in periodontal disease pathogenesis, Crit. Rev. Oral Biol. Med. 2 (1991) 177–281.
[8] W.E. Moore, Microbiology of periodontal disease, J. Periodontal Res. 22 (1987) 335–341.
[9] S. Persson, M.B. Edlund, R. Claesson, J. Carlsson, The formation of hydrogen sulfide and methyl mercaptan by oral bacteria, Oral Microbiol. Immunol. 5 (1990) 195–201.
[10] L. Chu, J.L. Ebersole, G.P. Kurzban, S.C. Holt, Cystalysin, a 46-kilodalton cysteine desulfhydrase from *Treponema denticola*, with hemolytic and hemoxidative activities, Infect. Immun. 65 (1997) 3231–3238.
[11] H.I. Krupka, R. Huber, S.C. Holt, T. Clausen, Crystal structure of cystalysin from *Treponema denticola*: a pyridoxal 5′-phosphate-dependent protein acting as a haemolytic enzyme, EMBO J. 19 (2000) 3168–3178.
[12] R.P. Blakemore, E. Canale-Parola, Arginine catabolism by *Treponema denticola*, J. Bacteriol. 128 (1976) 616–622.
[13] H. Hori, K. Takabayashi, L. Orvis, D.A. Carson, T. Nobori, Gene cloning and characterization of *Pseudomonas putida* L-methionine-α-deamino-γ-mercaptomethane-lyase, Cancer Res. 56 (1996) 2116–2122.
[14] H. Inoue, K. Inagaki, M. Sugimoto, N. Esaki, K. Soda, H. Tanaka, Structural analysis of the L-methionine γ-lyase gene from *Pseudomonas putida*, J. Biochem. (Tokyo) 117 (1995) 1120–1125.
[15] W. Kreis, C. Hession, Isolation and purification of L-methionine-α-deamino-γ-mercaptomethane-lyase (L-methioninase) from *Clostridium sporogenes*, Cancer Res. 33 (1973) 1862–1865.
[16] A.E. McKie, T. Edlind, J. Walker, J.C. Mottram, G.H. Coombs, The primitive protozoon *Trichomonas vaginalis* contains two methionine γ-lyase genes that encode members of the γ-family of pyridoxal 5′-phosphate-dependent enzymes, J. Biol. Chem. 273 (1998) 5549–5556.
[17] R. Claesson, M.B. Edlund, S. Persson, J. Carlsson, Production of volatile sulfur compounds by various *Fusobacterium* species, Oral Microbiol. Immunol. 5 (1990) 137–142.
[18] P.A. Ratcliff, P.W. Johnson, The relationship between oral malodor, gingivitis, and periodontitis. A review, J. Periodontol. 70 (1999) 485–489.
[19] K. Yaegaki, K. Sanada, Biochemical and clinical factors influencing oral malodor in periodontal patients, J. Periodontol. 63 (1992) 783–789.
[20] J.M. Coli, J. Tonzetich, Characterization of volatile sulphur compounds production at individual gingival crevicular sites in humans, J. Clin. Dent. 3 (1992) 97–103.
[21] T. Nakayama, N. Esaki, K. Sugie, T.T. Beresov, H. Tanaka, K. Soda, Purification of bacterial L-methionine γ-lyase, Anal. Biochem. 138 (1984) 421–424.
[22] G.H. Coombs, J.C. Mottram, Trifluoromethionine, a prodrug designed against methionine γ-lyase-containing pathogens, has efficacy in vitro and in vivo against *Trichomonas vaginalis*, Antimicrob. Agents Chemother. 45 (2001) 1743–1745.
[23] M. Korayem, G. Westbay, I. Kleinberg, Constituents of salivary supernatant responsible for stimulation of oxygen uptake by the bacteria in human salivary sediment, Arch. Oral Biol. 35 (1990) 145–152.
[24] R. Mashima, T. Nakanishi-Ueda, Y. Yamamoto, Simultaneous determination of methionine sulfoxide and methionine in blood plasma using gas chromatography-mass spectrometry, Anal. Biochem. 313 (2003) 28–33.