Identification and enzymic analysis of a novel protein associated with production of hydrogen sulfide and L-serine from L-cysteine in *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586

Yasuo Yoshida,¹ Kyosuke Suwabe,^{1,2} Keiji Nagano,¹ Yuichiro Kezuka,³ Hirohisa Kato⁴ and Fuminobu Yoshimura¹

Correspondence

Yasuo Yoshida
yasuoy@dpc.agu.ac.jp

¹Department of Microbiology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan

²Department of Conservative Dentistry and Oral Rehabilitation, School of Dentistry, Iwate Medical University, Morioka, Japan

³Department of Structural Biology, School of Pharmacy, Iwate Medical University, Yahaba, Japan

⁴Department of Pathogenesis and Control of Oral Disease, School of Dentistry, Iwate Medical University, Morioka, Japan

A third enzyme that produces hydrogen sulfide from L-cysteine was identified in *Fusobacterium nucleatum* subsp. *nucleatum*. The *fn1055* gene was cloned from a cosmid library constructed with genomic DNA of *F. nucleatum* ATCC 25586. Despite the database annotation that the product of *fn1055* is a cysteine synthase, reverse-phase HPLC revealed that no L-cysteine was produced *in vitro* by the purified Fn1055 protein; however, the enzyme did produce L-serine. In addition, a cysteine auxotroph, *Escherichia coli* NK3, transformed with a plasmid containing the *fn1055* gene did not grow without cysteine, which further suggests that Fn1055 does not function as a cysteine synthase. The Michaelis–Menten kinetics ($K_m=0.09\pm0.001$ mM and $k_{cat}=5.43\pm0.64$ s⁻¹) of the purified enzyme showed that the capacity of Fn1055 to produce hydrogen sulfide was between that of two other enzymes, Fn0625 and Fn1220. Incubation of Fn1055 with L-cysteine resulted in the production of hydrogen sulfide, but not of pyruvate, ammonia or lanthionine, which are all byproducts produced in addition to hydrogen sulfide when Fn0625 or Fn1220 is incubated with L-cysteine. Instead, Fn1055 produced L-serine in its reaction with L-cysteine. Fn1055 produces hydrogen sulfide from L-cysteine by a mechanism that is different from that of Fn0625 or Fn1220.

Received 11 February 2011

Revised 6 April 2011

Accepted 8 April 2011

INTRODUCTION

Hydrogen sulfide (H₂S) is a dominant volatile sulfur compound (VSC) in the human oral cavity, and VSCs are responsible for oral malodour (Tonzetich, 1971). H₂S not only causes aesthetic problems, but also has toxic effects on several tissues and host cells (Calenic *et al.* 2010a, b; Kurzban *et al.*, 1999; Li *et al.*, 2005; Morhart *et al.*, 1970; Ng & Tonzetich, 1984; Yaegaki *et al.*, 2008; Yang *et al.*, 2004; Yoshida *et al.*, 2002). In addition, the amount of H₂S in periodontal pockets is much higher than the levels of this compound (Persson, 1992) normally associated with the aetiology of periodontitis (Ratcliff & Johnson, 1999).

H₂S in the human oral cavity is produced as a metabolite of oral bacteria. *Fusobacterium nucleatum* is known to be a primary source of oral H₂S (Claesson *et al.*, 1990; Persson *et al.*, 1990; Pianotti *et al.*, 1986). This micro-organism has multiple enzymes that produce H₂S from L-cysteine (Persson *et al.*, 1990). Molecular analyses of Fn1220 (Cdl) and Fn0625 (Lcd), which are associated with H₂S production in *F. nucleatum*, have been recently reported (Fukamachi *et al.*, 2002; Yoshida *et al.*, 2010a). Fn1220 and Fn0625 produce H₂S in different ways (Yoshida *et al.*, 2010a): the former catalyses a β -replacement reaction in which two molecules of L-cysteine condense to generate H₂S and lanthionine, whereas the latter catalyses β -elimination of L-cysteine to produce H₂S, pyruvate and ammonia. Unsurprisingly, there is no significant amino acid sequence homology between these two enzymes. Even though crude enzyme extracts from *F. nucleatum* ATCC

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

25586 include at least three enzymes associated with production of H₂S from L-cysteine (Yoshida *et al.*, 2010a), the enzymes other than Fn1220 and Fn0625 remained to be identified.

In this study, a novel gene encoding an H₂S-producing enzyme was identified using a cosmid library constructed with the genomic DNA of *F. nucleatum* ATCC 25586. The enzymic properties of the 37 kDa enzyme encoded by the *fn1055* gene were studied. While Fn1055 also produced H₂S from L-cysteine, it differed from Fn1220 and Fn0625 as it produced L-serine as a byproduct.

METHODS

Bacterial strains and culture conditions. Bacteria used in this study are listed in Table 1. *F. nucleatum* subsp. *nucleatum* ATCC 25586 was grown anaerobically at 37 °C in Columbia broth (Difco). *Escherichia coli* strains were grown aerobically in LB medium (Difco), if necessary with 100 µg ampicillin ml⁻¹. *E. coli* NK3 (Noji *et al.*, 1994) was grown in M9-ALT, which is M9 minimal medium (Sambrook *et al.*, 1989) containing 100 µg ampicillin ml⁻¹, 200 µg leucine ml⁻¹ and 200 µg tryptophan ml⁻¹.

Preparation of crude enzyme extracts. Crude enzyme extracts of *F. nucleatum* ATCC 25586 and *E. coli* transformants were obtained as previously described (Yoshida *et al.*, 2010a). Briefly, each strain was grown to OD₆₀₀ ~0.8. The cells harvested from 40 ml of culture were washed three times with PBS (0.12 M NaCl, 0.01 M Na₂HPO₄, 5 mM KH₂PO₄, pH 7.5). The cell suspension was lysed by ultrasonication and centrifuged. Protein concentrations were determined using a protein assay reagent (Bio-Rad) with BSA as standard. The samples were stored at -20 °C after adding an equal volume of 80 % (v/v) glycerol.

Visualization of enzymic activity. The activity of crude or purified enzymes that produced H₂S from L-cysteine was visualized using SDS-PAGE with renaturation to remove the reducing/denaturing agent, followed by active staining, as previously described (Yoshida *et al.*, 2010b). Briefly, protein samples (20 µg for crude enzyme extracts and 2 µg for purified enzyme) were electrophoresed at 20 mA per gel at 4 °C on 12.5 % (w/v) polyacrylamide gels (pH 8.8) with

0.1 % SDS. The gels were renatured slowly in buffer by replacement of SDS with the detergents Triton X-100 and Lubrol PX so that the gels were shifted from noncatalytic to catalytic conditions. The gels were incubated in 10 ml of a visualizing solution [100 mM triethanolamine-HCl, pH 7.6, 10 µM pyridoxal 5'-phosphate (PLP), 0.5 mM bismuth trichloride, 10 mM EDTA, 5.0 mM L-cysteine] at 37 °C. The enzyme was detected as a black band with the appropriate apparent molecular mass (Claesson *et al.*, 1990).

Cloning of the gene encoding the H₂S-producing enzyme.

A cosmid gene bank of *F. nucleatum* ATCC 25586 was constructed as follows. After the chromosomal DNA of the strain had been partially digested with *ApoI*, approximately 5–15 kb fragments were obtained using sucrose density gradient ultracentrifugation (65 000 g), ligated with the 36 kb cosmid vector Charomid 9-36 (Nippon Gene) previously digested with *EcoRI*, packaged into bacteriophage λ (Gigapack III Gold Packaging Extracts, Stratagene), and then transfected into *E. coli* LC815, a mutant strain with a low capacity to produce H₂S from L-cysteine in a low-pH environment (Fukamachi *et al.*, 2002).

Approximately 2500 colonies in the cosmid gene bank were grown on 2 × TY plates, transferred to a nitrocellulose membrane, and soaked in visualizing solution as described above. The H₂S-producing colonies were dark brown.

Plasmids to produce recombinant proteins. Plasmids (Table 1) for production of proteins fused with glutathione S-transferase (GST) were constructed using the expression vector pGEX-6P-1 (GE Healthcare), as previously described (Yoshida *et al.*, 2002). Briefly, each entire gene was PCR-amplified with primers designed to incorporate a *Bam*HI site at the 5' end and a *Sal*I site at the 3' end of each segment. After PCR amplification, the reaction products were digested with *Bam*HI and *Sal*I and ligated into pGEX-6P-1, juxtaposing the gene fragment downstream from the coding sequence for GST and a PreScission protease cleavage site to yield pGEX6p-1-*fn1055*.

Purification of recombinant proteins. To purify the *fn1055* protein product, pGEX6p-1-*fn1055* was transformed into *E. coli* BL21, which was grown to OD₆₀₀ 0.7 at 37 °C, and 0.2 l cultures were induced with 0.3 mM IPTG for 2 h. Cells were centrifuged at 5000 g, resuspended in 20 ml PBS, and then sonicated. Cell debris was removed by centrifugation at 20 000 g and 4 °C. The supernatant was loaded onto a 1 ml glutathione Sepharose 4B (GE Healthcare)

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>F. nucleatum</i> subsp. <i>nucleatum</i> ATCC 25586	Isolate from a cervico-facial lesion	RIKEN BioResource Center, Wako, Japan
<i>E. coli</i> DH5α	F ⁻ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA96 φ80lacZM15</i>	Invitrogen
<i>E. coli</i> BL21	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm rne131</i>	Novagen
<i>E. coli</i> LC815	Mutant strain with a low activity for H ₂ S production	Fukamachi <i>et al.</i> (2002)
<i>E. coli</i> NK3	Δ <i>trpE5 leu-6 thi hsdR hsdM</i> ⁺ <i>cysK cysM</i>	Noji <i>et al.</i> (1994)
Plasmids		
pGEX6P-1	Cloning vector	GE Healthcare
pGEX6p-1- <i>cysM</i>	Production of GST-CysM of <i>E. coli</i> K-12	This study
pGEX6p-1- <i>cysK</i>	Production of GST-CysK of <i>E. coli</i> K-12	This study
pGEX6p-1- <i>fn0625</i>	Production of GST-Fn0625 of <i>F. nucleatum</i> ATCC 25586	Yoshida <i>et al.</i> (2010a)
pGEX6p-1- <i>fn1055</i>	Production of GST-Fn1055 of <i>F. nucleatum</i> ATCC 25586	This study
pGEX6p-1- <i>fn1220</i>	Production of GST-Fn1220 of <i>F. nucleatum</i> ATCC 25586	Yoshida <i>et al.</i> (2010a)

column equilibrated with PBS. The resin was washed with PBS, equilibrated with PreScission protease buffer (50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT), and incubated with 80 U PreScission protease (GE Healthcare) for 12 h at 4 °C. The enzyme was eluted in 2 ml PreScission protease buffer. GST and PreScission protease remain bound to the beads in this procedure. The eluate was loaded onto a prepacked anion exchange column (HiTrap Q HP, GE Healthcare), a linear NaCl gradient (0.1–0.6 M) in 20 mM Tris/HCl buffer, pH 8.5, was used for elution at a flow rate of 0.5 ml min⁻¹ at 4 °C, and 1.0 ml fractions were collected. The enzyme samples were stored at -20 °C after adding an equal volume of 80 % (v/v) glycerol. The protein concentrations were determined as described by Pace *et al.* (1995). The purity of the protein samples was assessed by SDS-PAGE.

The Fn0625 and Fn1220 proteins were prepared as described previously (Yoshida *et al.*, 2010a), and were further purified using the anion exchange HiTrap Q HP column described above.

Gel-filtration chromatography. The molecular mass of the purified Fn1055 proteins was determined by gel-filtration chromatography with a Superdex 200 HR 10/30 column (GE Healthcare) at a flow rate of 0.25 ml min⁻¹ in PBS. A standard curve was produced by gel filtration of molecular mass standards (kit for molecular weights 43 000–158 000, GE Healthcare). Enzyme elution was monitored at 280 nm. At least two independent experiments were performed, and a representative chromatogram is shown.

Enzyme assay. The enzymic activity of the purified proteins was assessed using a modification of the method of Schmidt (1987), which measures the production of H₂S from L-cysteine. Briefly, the reaction mixture (100 µl) contained 40 mM potassium phosphate buffer (pH 7.6), 10 µM PLP, the purified enzyme (Fn1055, 159.5 ng; Fn1220, 101 ng; Fn0625, 4.65 µg), and various concentrations of substrate(s). The reaction (100 µl) was incubated at 37 °C for 2 min, and terminated by the addition of 20 µl termination buffer, which was freshly prepared by mixing 10 µl solution I (20 mM *N,N'*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl) and 10 µl solution II (30 mM FeCl₃ in 1.2 M HCl). After incubation for 30 min at room temperature, methylene blue formation was measured spectrophotometrically at 670 nm alongside a standard curve. The standard curve was prepared using βC-S lyase from *Streptococcus anginosus* IMU102, which produces equal amounts of pyruvate and H₂S from L-cysteine (Ito *et al.*, 2008).

Kinetic parameters were computed from the Lineweaver–Burk transformation of the Michaelis–Menten equation. The *k*_{cat} values were calculated using the *V*_{max} values and the molecular masses of the enzymes.

The formation of ammonia as an end product was determined using an Ammonia-test_{wako} kit (Wako Pure Chemical) following the manufacturer's protocols.

Gas chromatography. Gas chromatography was performed as previously described (Yoshida *et al.*, 2010a) to detect sulfur compounds, including H₂S, methylmercaptan and ethylmercaptan (ethanethiol), which were predicted to be byproducts formed upon β- or γ-elimination of specific substrates. The reaction mixture (100 µl) contained 20 µg recombinant protein, 10 mM substrate, 10 µM PLP and 40 mM potassium phosphate buffer (pH 7.6). The reaction mixture was prepared in a 10 ml glass tube, sealed with a silicone plug, and incubated at 37 °C for 10 min. A sample (1 ml) of the vapour above the reaction mixture was removed with a gas-tight syringe, without removing the silicone plug, and analysed by gas chromatography (model GC-8A, Shimadzu) using a Teflon column packed with 20 % β,β'-oxydipropionitrile on an 80–100 mesh Chromosorb W AW-DMCS-ST device (Shimadzu) fitted with a flame photometric detector at 70 °C.

HPLC. The production of serine, homoserine, homocysteine and 2-aminoethanethiol, which were predicted to be products of Fn1055, was assessed using a reverse-phase HPLC column. The reaction mixtures contained the following reagents in a final volume of 100 µl: 40 mM potassium phosphate buffer (pH 8.5), 10 µM PLP, 5 mM substrate(s) and 2.5 µg purified enzyme. After the mixtures had incubated for 2.5 h at 37 °C, the enzymes were removed using a Microcon YM-10 filter (10 kDa cut-off, Amicon). The ultrafiltration products were dansylated as described by Tapuhi *et al.* (1981). Aliquots (20 µl) of each sample were applied to an XTerra RP₁₈ column by injection (4.6 × 150 mm, Waters). The dansylated products were separated at a flow rate of 1.0 or 0.5 ml min⁻¹ at 40 °C with a mobile phase of 33.25/66.75 (v/v) methanol/water containing 0.6 % (v/v) glacial acetic acid and 0.008 % (v/v) triethylamine. Excitation and emission wavelengths were 350 and 530 nm, respectively. At least two independent experiments were performed, and representative chromatograms are shown.

Growth test using the cysteine auxotroph *E. coli* NK3. The cysteine auxotroph *E. coli* NK3 was transformed with the plasmids listed in Table 1. The transformants grown in LB broth for 24 h were washed three times with M9-ALT, plated on M9-ALT agar, and then incubated for 18 h.

RESULTS

Cloning of *fn1055*, encoding a new enzyme associated with H₂S production

Previous results (Yoshida *et al.*, 2010b) showed that crude enzyme extracts from each of five subspecies of *F. nucleatum* contained multiple H₂S-producing enzymes. These included Fn0625 and Fn1220, which have been characterized previously (Fukamachi *et al.*, 2002; Yoshida *et al.*, 2010a). To identify additional enzymes that produce H₂S from L-cysteine, a cosmid library was constructed with the genomic DNA of *F. nucleatum* ATCC 25586. Of 2500 colonies tested, seven that produced H₂S were identified by their brown colour in the visualizing solution. PCR analysis resulted in amplification of *fn1220* from four positive colonies, but neither *fn1220* nor *fn0625* was detected in the remaining three colonies. Since those three cosmid DNAs had identical restriction enzyme fingerprinting profiles, one representative cosmid designated pLC10-1-21 was used for analysis. Activity staining analysis combined with SDS-PAGE, which was followed by renaturation, confirmed that the crude enzyme extracts from *E. coli* harbouring pLC10-1-21 contained one protein band associated with H₂S production (Fig. 1a, lane 3). This indicated that pLC10-1-21 contained a gene encoding an H₂S-producing protein.

DNA sequence analysis showed that pLC10-1-21 contained a 10 kb DNA fragment with the *fn1055* gene that encodes a putative cysteine synthase (Kapatral *et al.*, 2002). No other genes predicted to be associated with the assimilation and catabolism of sulfur-containing molecules were identified within that DNA. The amino acid sequence of the Fn1055 protein showed 30 and 32 % identity with that of *E. coli* CysM and CysK, respectively. Both of these enzymes synthesize L-cysteine from O-acetylserine and sulfide (Kredich, 1996). Fn1055 also showed amino acid sequence

Fig. 1. Analysis of crude enzyme extracts and purified proteins of *F. nucleatum* ATCC 25586. (a) SDS-PAGE analysis. Left panel, gel stained with Coomassie brilliant blue; right panel, after the gel was renatured (see Methods), the enzymic activity to produce H₂S was visualized by active staining. Lanes: 1, cell lysate from *F. nucleatum* subsp. *nucleatum* ATCC 25586; 2, cell lysate from *E. coli* LC815 harbouring a cosmid vector, charomid 9-36 (negative control); 3, cell lysate from *E. coli* LC815 harbouring pLC10-1-21; 4, purified Fn1055 of *F. nucleatum* subsp. *nucleatum* ATCC 25586; 5, purified Fn0625 of *F. nucleatum* subsp. *nucleatum* ATCC 25586; 6, purified Fn1220 of *F. nucleatum* subsp. *nucleatum* ATCC 25586. The amounts of crude and purified proteins applied to the gel were 20 and 1 µg, respectively. The value for each molecular mass marker is given in kDa. (b) Gel-filtration chromatography of purified Fn1055 on Superdex 200 HR10/30. The elution volumes of molecular mass standards (158 kDa, aldolase; 75 kDa, conalbumin; 43 kDa ovalbumin) are indicated by arrows.

identity (33%) with Fn1220 but not with Fn0625 in *F. nucleatum* ATCC 25586 (Yoshida *et al.*, 2010a).

Purification and characterization of Fn1055

To evaluate the enzymic activity of Fn1055, the recombinant protein was purified (Fig. 1a, lane 4). The molecular mass of the denatured polypeptide was approximately

37 kDa, which agreed with the predicted molecular mass (37.3 kDa). Gel-filtration chromatography showed that the purified Fn1055 was eluted at retention volumes corresponding to 75 kDa conalbumin (Fig. 1b), suggesting that Fn1055 protein may exist as a homodimer in solution. Activity staining analysis (Fig. 1a) showed that the purified Fn1055 protein (lane 4) produced H₂S from L-cysteine. Furthermore, the electrophoretic migration of the recombinant protein agreed with that observed for the H₂S-producing protein found in the crude enzyme extracts from *E. coli* that harboured pLC10-1-21 (lane 3).

Fn1055 produces serine from cysteine

Gas chromatography analysis confirmed that incubation of recombinant Fn1055 with L-cysteine yielded H₂S (data not shown). Spectrophotometric analyses did not identify the presence of pyruvate or ammonia, which are produced as byproducts in the reaction of Fn0625 with L-cysteine. Furthermore, HPLC analysis did not detect lanthionine, a byproduct of the reaction of Fn1220 with L-cysteine. These results suggested that Fn1055 might convert L-cysteine to L-serine and H₂S. HPLC analysis demonstrated that the reaction of Fn1055 with dansylated L-cysteine yielded dansylated L-serine (Fig. 2).

Kinetic properties of Fn1055

A spectrophotometric assay that measured the production of H₂S in the presence of L-cysteine was used to determine

Fig. 2. Reverse-phase HPLC analysis of Fn1055 reaction products. Dansylated reaction products were detected using excitation and emission wavelengths of 350 and 530 nm, respectively. Top panel, reaction products obtained without Fn1055 (negative control); bottom panel, reaction products obtained with purified Fn1055. Peaks were identified by retention time.

Table 2. Kinetic properties of Fn1055, Fn0625 and Fn1220 from *F. nucleatum* ATCC 25586

Enzyme	End products	K_m (mM)	k_{cat} (s^{-1})
Fn1055	H ₂ S, serine	0.09 ± 0.001	5.43 ± 0.64
Fn0625	H ₂ S, ammonia, pyruvate	0.152 ± 0.017	0.092 ± 0.009
Fn1220	H ₂ S, lanthionine	0.156 ± 0.012	17.07 ± 0.941

the activities of the purified Fn1055, Fn0625 and Fn1220 proteins (Table 2). The K_m value for Fn1055 was lower than the values determined for Fn0625 and Fn1220, suggesting that Fn1055 has a higher affinity for L-cysteine. By contrast, the k_{cat} value for Fn1055 was lower than that for Fn1220, but was higher than that for Fn0625. To evaluate the enzymic properties of Fn1055, several sulfur-containing molecules were tested for β - and γ -elimination (Table 3). With the exception of L-cysteine, none of the molecules listed in Table 3 was a substrate for Fn1055.

Fn1055 does not promote synthesis of cysteine

Two assays were used to determine whether Fn1055 promotes synthesis of L-cysteine. First, unlike CysK and CysM in *E. coli* (Sirko *et al.*, 1987; Wiater & Hulanicka, 1979), Fn1055 did not produce L-cysteine from O-acetylserine and NaHS (Fig. 3a). Second, incubation of Fn1055 with L-serine and NaHS did not yield L-cysteine (data not shown). Furthermore, the cysteine auxotroph *E. coli* NK was transformed with pGEX6p-1-*fn1055* (Table 1), which encoded a fusion protein of GST and Fn1055. The transformant did not grow on M9 minimal medium without L-cysteine, which provided additional evidence that Fn1055 does not catalyse biosynthesis of L-cysteine. *E. coli* NK3 did not grow in the absence of L-cysteine (Fig. 3b) when transformed with pGEX6p-1-*fn0625* or pGEX6p-1-*fn1220* (Table 1), but it grew well when transformed with either pGEX6p-1-*cysM* or pGEX6p-1-*cysK*.

DISCUSSION

F. nucleatum has a high H₂S-producing activity (Persson *et al.*, 1990). Five subspecies of *F. nucleatum* (i.e. *nucleatum*, *vincentii*, *polymorphum*, *fusiforme* and *animalis*) each contain multiple enzymes that produce H₂S from L-cysteine (Yoshida *et al.*, 2010b), and two of these H₂S-producing enzymes (i.e. Fn0625 and Fn1220) have been characterized (Yoshida *et al.*, 2010a). The current study of *F. nucleatum* subsp. *nucleatum* identified Fn1055 as another H₂S-producing enzyme.

Of seven H₂S-producing colonies identified from the cosmid library constructed with the genomic DNA of *F. nucleatum* ATCC 25586, three carried the *fn1055* gene, whereas the remaining four carried the *fn1220* gene. No colonies carrying the *fn0625* gene were isolated, probably because the H₂S-producing activity of Fn0625 is much lower than those of Fn1055 and Fn1220 (Table 2). We also found no colonies containing other H₂S-producing proteins, including the approximately 130 kDa protein, which seemed to have the highest activity to produce H₂S in the crude enzyme extracts of *F. nucleatum* ATCC 25586 (Fig. 1b). It would be of interest to identify and characterize this protein, which was observed to be common to five subspecies of *F. nucleatum* (Yoshida *et al.*, 2010b).

The K_m of Fn1055 was the lowest among the three H₂S-producing enzymes in *F. nucleatum* ATCC 25586, suggesting that Fn1055 has the highest affinity for L-cysteine (Table 2). By contrast, the k_{cat} value of Fn1055 was approximately one-third of that found for Fn1220, but was much higher than that found for Fn0625. Previously reported kinetic values for Fn0625 and Fn1220 (Yoshida *et al.*, 2010a) were different from those determined in this study (Table 2). The discrepancy might be caused by differences in methods of enzyme purification and spectrophotometric assays. In the current study, an anion-exchange column and a GST affinity column were used to obtain more highly purified enzymes, while in the

Table 3. Potential elimination reactions catalysed by Fn1055 from *F. nucleatum* ATCC 25586

Reaction	Substrate	β - or γ -Elimination reaction			
		Elimination type	Predicted end products		Catalysed by Fn1055
1	L-Cysteine	β	Serine*	H ₂ S†	Yes
2	L-Cystathionine	β	Serine	Homocysteine*	No
3	S-(2-Aminoethyl)-L-cysteine	β	Serine	2-Aminoethanethiol (cysteamine)*	No
4	S-Methyl-L-cysteine	β	Serine	Methylmercaptan†	No
5	S-Ethyl-L-cysteine	β	Serine	Ethylmercaptan† (ethanethiol)	No
6	Methionine	γ	Homoserine*	Methylmercaptan†	No

*Determined by HPLC.

†Determined by gas chromatography.

Fig. 3. Fn1055 is not a cysteine synthase. (a) Reverse-phase HPLC analysis for L-cysteine in the reactions of O-acetyl-L-serine and NaHS with purified Fn1055. Top panel, reaction products obtained without Fn1055 (negative control); bottom panel, reaction products obtained with purified Fn1055. (b) Genetic complementation analysis of the cysteine auxotroph *E. coli* NK3, which was transformed with (1) pGEX-6p-1 (negative control), (2) pGEX-6p-1-*cysM*, (3) pGEX-6p-1-*cysK*, (4) pGEX-6p-1-*fn0625*, (5) pGEX-6p-1-*fn1055* and (6) pGEX-6p-1-*fn1220*. The transformants were spread on M9 minimal agar plates supplemented with 100 µg ampicillin ml⁻¹, 200 µg leucine ml⁻¹ and 100 µg tryptophan ml⁻¹, with or without 0.5 mM L-cysteine.

previous study only the GST affinity column was used. In addition, the original method to measure H₂S using methylene blue was modified for the present study. In previous studies, the terminating and developing solutions were added to the reaction mixtures separately (Yoshida *et al.*, 2010a). In this study, the two solutions were added at the same time, which allowed the H₂S produced to react completely with the reagent. Thus, the kinetic measurements of Fn0625 and Fn1220 might be more precise here than in earlier studies.

In *E. coli* and several other micro-organisms, L-serine is biosynthesized from the glycolytic intermediate 3-phosphoglycerate by sequential reactions of 3-phosphoglycerate dehydrogenase, phosphoserine aminotransferase and phosphoserine phosphatase. Interestingly, a database analysis showed that the genomic DNA of *F. nucleatum* ATCC 25586 does not contain any homologous genes that encode these enzymes. However, as shown in this study, L-serine

was produced in the reaction of Fn1055 with L-cysteine. Although it is not known whether β -elimination of L-cysteine is the only pathway to produce L-serine in *F. nucleatum*, the biosynthetic pathways of amino acids, including L-serine, seem to be different from those reported in *E. coli* and other bacteria. Further investigation is necessary to understand the assimilation and catabolism of amino acids in *F. nucleatum*.

Three *F. nucleatum* enzymes (i.e. Fn1055, Fn0625 and Fn1220), all of which form homodimers in solution (Yoshida *et al.*, 2010a), are associated with H₂S production from L-cysteine, but all have distinct mechanisms. Considering the bacterial pathways for H₂S production, most reactions reported so far involve α,β -elimination of L-cysteine, in which pyruvate and ammonia are produced together with H₂S. This reaction is associated with the Fn0625 protein in *F. nucleatum* (Fig. 4). Other bacterial pathways that produce H₂S have only recently been

Fn01055 : β -Elimination **Fn0625** : α,β -Elimination **Fn1220** : β -Replacement **Fig. 4.** Reactions catalysed by Fn1055, Fn0625 and Fn1220.

reported (Yoshida *et al.*, 2010a), and involve β -replacement of L-cysteine catalysed by Fn1220 (Fig. 4). Thus, β -elimination to produce H_2S and serine from L-cysteine may be a novel finding in bacteria. By contrast, human cystathionine β -synthase (CBS), which converts L-serine and homocysteine to L-cystathionine, also catalyses β -elimination of L-cysteine to produce L-serine and H_2S as well as β -replacement of L-cysteine to produce L-lanthionine and H_2S (Singh *et al.*, 2009). In *F. nucleatum*, the former reaction was catalysed by Fn1055 whereas the latter reaction was catalysed by Fn1220. However, the CBS turnover number for serine production is approximately 3 % of that found for cystathionine biosynthesis, suggesting that degradation of L-cysteine by CBS might be an incidental reaction due to its broad substrate specificity.

It is not known why H_2S production is mediated by three different enzymes in *F. nucleatum*. The capacity of Fn0625 to produce L-homocysteine from L-cystathionine is much higher than its capacity to produce H_2S from L-cysteine (Yoshida *et al.*, 2010a). Since L-homocysteine is an intermediate in the L-methionine biosynthetic pathway, Fn0625 may be important in terms of L-methionine biosynthesis. In contrast, the substrate specificities of

Fn1055 and Fn1220 seem to be limited to L-cysteine only (Yoshida *et al.*, 2010a). The byproducts (i.e. L-serine and L-lanthionine) in the reaction of the proteins with L-cysteine might be more important in *F. nucleatum*. Indeed, lanthionine is a natural constituent of the peptidoglycans isolated from *F. nucleatum* (Kato *et al.*, 1979).

In conclusion, a third enzyme (Fn1055) associated with H_2S production in *F. nucleatum* was identified and characterized. The mechanism used to produce H_2S from L-cysteine was found to be different from those of previously characterized enzymes. However, the approximately 130 kDa protein showing the highest activity to produce H_2S from L-cysteine in the crude enzyme extracts of *F. nucleatum* ATCC 25586 has not been elucidated. Further studies are necessary to understand the mechanism of H_2S production in *F. nucleatum*.

ACKNOWLEDGEMENTS

We thank Dr Yoshio Nakano of Nihon University (Tokyo, Japan) and Dr Kazuki Saito of Chiba University (Chiba, Japan) for providing *E. coli* strains LC815 and NK3, respectively. This study was supported in part by Grants-in-Aid for Scientific Research (nos 20592463 and

20592181) and by the Strategic Medical Research Center of the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work is dedicated to people in the Iwate prefecture who lost their lives in the earthquake and tsunami on 11 March 2011.

REFERENCES

- Calenic, B., Yaegaki, K., Kozuharova, A. & Imai, T. (2010a). Oral malodorous compound causes oxidative stress and p53-mediated programmed cell death in keratinocyte stem cells. *J Periodontol* **81**, 1317–1323.
- Calenic, B., Yaegaki, K., Murata, T., Imai, T., Aoyama, I., Sato, T. & Ii, H. (2010b). Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in human gingival epithelial cells. *J Periodontol Res* **45**, 31–37.
- Claesson, R., Edlund, M. B., Persson, S. & Carlsson, J. (1990). Production of volatile sulfur compounds by various *Fusobacterium* species. *Oral Microbiol Immunol* **5**, 137–142.
- Fukamachi, H., Nakano, Y., Yoshimura, M. & Koga, T. (2002). Cloning and characterization of the L-cysteine desulphydrase gene of *Fusobacterium nucleatum*. *FEMS Microbiol Lett* **215**, 75–80.
- Ito, S., Nagamune, H., Tamura, H. & Yoshida, Y. (2008). Identification and molecular analysis of β C-S lyase producing hydrogen sulfide in *Streptococcus intermedius*. *J Med Microbiol* **57**, 1411–1419.
- Kapatral, V., Anderson, I., Ivanova, N., Reznik, G., Los, T., Lykidis, A., Bhattacharyya, A., Bartman, A., Gardner, W. & other authors (2002). Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J Bacteriol* **184**, 2005–2018.
- Kato, K., Umemoto, T., Sagawa, H. & Kotani, S. (1979). Lanthionine as an essential constituent of cell wall peptidoglycan of *Fusobacterium nucleatum*. *Curr Microbiol* **3**, 147–151.
- Kredich, N. M. (1996). Biosynthesis of cysteine. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 514–527. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- Kurzban, G. P., Chu, L., Ebersole, J. L. & Holt, S. C. (1999). Sulfhemoglobin formation in human erythrocytes by cystalysin, an L-cysteine desulphydrase from *Treponema denticola*. *Oral Microbiol Immunol* **14**, 153–164.
- Li, L., Bhatia, M., Zhu, Y. Z., Zhu, Y. C., Ramnath, R. D., Wang, Z. J., Anuar, F. B., Whiteman, M., Salto-Tellez, M. & Moore, P. K. (2005). Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J* **19**, 1196–1198.
- Morhart, R. E., Mata, L. J., Sinskey, A. J. & Harris, R. S. (1970). A microbiological and biochemical study of gingival crevice debris obtained from Guatemalan Mayan Indians. *J Periodontol* **41**, 644–649.
- Ng, W. & Tonzetich, J. (1984). Effect of hydrogen sulfide and methyl mercaptan on the permeability of oral mucosa. *J Dent Res* **63**, 994–997.
- Noji, M., Murakoshi, I. & Saito, K. (1994). Molecular cloning of a cysteine synthase cDNA from *Citrullus vulgaris* (watermelon) by genetic complementation in an *Escherichia coli* Cys[−] auxotroph. *Mol Gen Genet* **244**, 57–66.
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* **4**, 2411–2423.
- Persson, S. (1992). Hydrogen sulfide and methyl mercaptan in periodontal pockets. *Oral Microbiol Immunol* **7**, 378–379.
- Persson, S., Edlund, M. B., Claesson, R. & Carlsson, J. (1990). The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* **5**, 195–201.
- Pianotti, R., Lachette, S. & Dills, S. (1986). Desulfuration of cysteine and methionine by *Fusobacterium nucleatum*. *J Dent Res* **65**, 913–917.
- Ratcliff, P. A. & Johnson, P. W. (1999). The relationship between oral malodor, gingivitis, and periodontitis. A review. *J Periodontol* **70**, 485–489.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmidt, A. (1987). D-Cysteine desulphydrase from spinach. In *Methods Enzymol*, pp. 449–451. Edited by W. Jakoby & O. Griffith. New York: Academic Press.
- Singh, S., Padovani, D., Leslie, R. A., Chiku, T. & Banerjee, R. (2009). Relative contributions of cystathionine β -synthase and γ -cystathionase to H₂S biogenesis via alternative trans-sulfuration reactions. *J Biol Chem* **284**, 22457–22466.
- Sirko, A. E., Zatyka, M. & Hulanicka, M. D. (1987). Identification of the *Escherichia coli* *cysM* gene encoding O-acetylserine sulphydrylase B by cloning with mini-Mu-lac containing a plasmid replicon. *J Gen Microbiol* **133**, 2719–2725.
- Tapuhi, Y., Schmidt, D. E., Lindner, W. & Karger, B. L. (1981). Dansylation of amino acids for high-performance liquid chromatography analysis. *Anal Biochem* **115**, 123–129.
- Tonzetich, J. (1971). Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Arch Oral Biol* **16**, 587–597.
- Wiater, A. & Hulanicka, D. (1979). Properties of *cysK* mutants of *Escherichia coli* K12. *Acta Biochim Pol* **26**, 21–28.
- Yaegaki, K., Qian, W., Murata, T., Imai, T., Sato, T., Tanaka, T. & Kamoda, T. (2008). Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. *J Periodontol Res* **43**, 391–399.
- Yang, G., Sun, X. & Wang, R. (2004). Hydrogen sulfide-induced apoptosis of human aorta smooth muscle cells via the activation of mitogen-activated protein kinases and caspase-3. *FASEB J* **18**, 1782–1784.
- Yoshida, Y., Nakano, Y., Amano, A., Yoshimura, M., Fukamachi, H., Oho, T. & Koga, T. (2002). *lcd* from *Streptococcus anginosus* encodes a C-S lyase with α,β -elimination activity that degrades L-cysteine. *Microbiology* **148**, 3961–3970.
- Yoshida, Y., Ito, S., Kamo, M., Kezuka, Y., Tamura, H., Kunimatsu, K. & Kato, H. (2010a). Production of hydrogen sulfide by two enzymes associated with biosynthesis of homocysteine and lanthionine in *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586. *Microbiology* **156**, 2260–2269.
- Yoshida, Y., Ito, S., Tamura, H. & Kunimatsu, K. (2010b). Use of a novel assay to evaluate enzymes that produce hydrogen sulfide in *Fusobacterium nucleatum*. *J Microbiol Methods* **80**, 313–315.

Edited by: D. Demuth