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Production of hydrogen sulfide by two enzymes associated with biosynthesis of homocysteine and lanthionine in *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586

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Fusobacterium nucleatum produces a large amount of the toxic metabolite hydrogen sulfide in the oral cavity. Here, we report the molecular basis of *F. nucleatum* H_2S production, which is associated with two different enzymes: the previously reported Cdl (Fn1220) and the newly identified Lcd (Fn0625). SDS-PAGE analysis with activity staining revealed that crude enzyme extracts from *F. nucleatum* ATCC 25586 contained three major H_2S-producing proteins. Two of the proteins with low molecular masses migrated similarly to purified Fn0625 and Fn1220. Their kinetic values suggested that Fn0625 had a lower enzymic capacity to produce H_2S from L-cysteine (~30 %) than Fn1220. The Fn0625 protein degraded a variety of substrates containing βC–S linkages to produce ammonia, pyruvate and sulfur-containing products. Unlike Fn0625, Fn1220 produced neither pyruvate nor ammonia from L-cysteine. Reversed-phase HPLC separation and mass spectrometry showed that incubation of L-cysteine with Fn1220 produced H_2S and an uncommon amino acid, lanthionine, which is a natural constituent of the peptidoglycans of *F. nucleatum* ATCC 25586. In contrast, most of the sulfur-containing substrates tested, except L-cysteine, were not used by Fn1220. Real-time PCR analysis demonstrated that the *fn1220* gene showed several-fold higher expression than *fn0625* and housekeeping genes in exponential-phase cultures of *F. nucleatum*. Thus, we conclude that Fn0625 and Fn1220 produce H_2S in distinct manners: Fn0625 carries out β-elimination of L-cysteine to produce H_2S, pyruvate and ammonia, whereas Fn1220 catalyses the β-replacement of L-cysteine to produce H_2S and lanthionine, the latter of which may be used for peptidoglycan formation in *F. nucleatum*.

INTRODUCTION

Hydrogen sulfide (H_2S) is a gas with the smell of rotten eggs and is one of the predominant volatile sulfur compounds primarily responsible for halitosis (Tonzetich, 1971). Oral malodour appears to be associated with an increase in the number of H_2S-producing micro-organisms among tongue biofilm microflora (Washio *et al.*, 2005). It has been demonstrated that, *in vitro*, H_2S damages epithelial cells (Morhart *et al.*, 1970) and increases the permeability of oral mucosa (Ng & Tonzetich, 1984). More recently, it was reported that H_2S is associated with the modification and release of haemoglobin in erythrocytes (Kurzban *et al.*, 1999; Yoshida *et al.*, 2002), endotoxin-induced inflammation (Li *et al.*, 2005), and apoptosis of aorta smooth muscle cells (Yang *et al.*, 2004) and human gingival fibroblasts (Yaegaki *et al.*, 2008). An increasing amount of evidence is demonstrating that extremely low concentrations of H_2S are highly toxic to tissues and host cells, although most sufferers perceive halitosis primarily as a cosmetic problem. When considering the toxicity of H_2S, it is of interest that the amount of H_2S in periodontal pockets (Persson, 1992) is much higher than the levels of this compound that are normally associated with the aetiology of periodontitis (Ratcliff & Johnson, 1999).

Abbreviation: PLP, pyridoxal 5′-phosphate.

Fusobacterium nucleatum, the most abundant Gram-negative bacterium isolated from dental plaque biofilms (Moore & Moore, 1994), is a central species in biofilm development and a pathogen in human infections, including gingivitis and periodontitis (Kolenbrander *et al.*, 2002). This micro-organism is also known to be one of the most active oral bacteria in terms of H_2S production (Claesson *et al.*, 1990; Persson *et al.*, 1990; Pianotti *et al.*, 1986). Interestingly, multiple forms of H_2S-producing enzymes with large variations in electrophoretic mobility were found among several strains of *F. nucleatum* (Claesson *et al.*, 1990). The *cdl* gene in *F. nucleatum* subsp. *polymorphum* ATCC 10953 encodes a 33 kDa protein that produces H_2S from L-cysteine (Fukamachi *et al.*, 2002). The precise mechanism of H_2S production by this enzyme remains to be elucidated, although the protein was predicted to have βC–S lyase activity, catalysing the α,β-elimination of L-cysteine to produce H_2S, pyruvate and ammonia. The amino acid sequence of the Cdl protein of strain ATCC 10953 shows little identity with βC–S lyase proteins in several micro-organisms (Fukamachi *et al.*, 2002). No genes encoding H_2S-producing enzymes, other than *cdl*, have been identified in *F. nucleatum* so far.

In this study, a 47 kDa protein showing a high identity to βC–S lyase, which is encoded by the *fn0625* gene, was purified from *F. nucleatum* subsp. *nucleatum* ATCC 25586, and then characterized. We also purified a 33 kDa protein encoded by the *cdl* homologue (*fn1220*) of strain ATCC 25586 to evaluate its enzymic properties. In the process of characterizing the enzymic properties of Fn0625 and Fn1220, it was discovered that these enzymes produce H_2S in distinct manners.

METHODS

Bacterial strains and culture conditions. *F. nucleatum* subsp. *nucleatum* ATCC 25586 and subsp. *polymorphum* ATCC 10953 were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The strains were grown anaerobically at 37 °C in Columbia broth (Difco). *Escherichia coli* strains DH5α (Invitrogen) and BL21 (Promega) were used for DNA manipulation and protein purification, respectively, and were grown aerobically in 2× TY broth (Difco) at 37 °C. When required, 100 μg ampicillin ml^{-1} was added to the media.

Preparation of crude enzyme extracts. Each strain of *F. nucleatum* was grown to an OD_{600} of ~0.8. The cells were then harvested from 40 ml of culture and washed with PBS (0.12 M NaCl, 0.01 M Na_2HPO_4, 5 mM KH_2PO_4, pH 7.5) three times. A 1.0 ml aliquot of the cell suspension was lysed by ultrasonication and the supernatant was centrifuged. The protein concentrations were determined using a protein assay reagent (Bio-Rad) with BSA as a standard. The samples were stored at −20 °C after adding an equal volume of 80 % (v/v) glycerol.

Visualization of enzymic activities. H_2S production from L-cysteine by crude or purified enzymes was visualized by SDS-PAGE with renaturation to remove the reducing/denaturing agent, followed by activity staining, as described recently (Yoshida *et al.*, 2010). Samples were electrophoresed at 10 mA per gel at 4 °C on 12.5 % (w/v) polyacrylamide gels (pH 8.8) with 0.1 % SDS. To shift the gel from noncatalytic to catalytic conditions, the gels were renatured slowly in buffer by replacement of SDS with the detergents Triton X-100 and Lubrol PX. To detect the positions of the enzymes, the gels were incubated in visualizing solution (100 mM triethanolamine.HCl, pH 7.6, 10 μM pyridoxal 5′-phosphate (PLP), 0.5 mM bismuth trichloride, 10 mM EDTA and 5.0 mM L-cysteine) at 37 °C (Claesson *et al.*, 1990).

Purification of recombinant proteins encoded by *fn0625* and *fn1220*. Recombinant enzymes encoded by *fn0625* and *fn1220* in *F. nucleatum* ATCC 25586 were purified using the expression vector pGEX-6P-1 (GE Healthcare), as previously described (Yoshida *et al.*, 2002). Briefly, each gene was amplified by PCR using primers designed to incorporate a *Bam*HI site at the 5′ end and a *Sal*I site at the 3′ end of each segment (Table 1). Following PCR amplification, the 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 glutathione *S*-transferase and a PreScission protease cleavage site. The recombinant *E. coli* clones were diluted 1/1000 in 2× YT (500 ml) and cultured at 37 °C until the OD_{600} reached 0.5–0.7. Protein expression was induced by the addition of IPTG to a final concentration of 0.3 mM, and the temperature was maintained at 37 °C. The cells were harvested by centrifugation after 2 h of induction, then they were sonicated in PBS, and the cell debris was removed by centrifugation at 15 000 r.p.m. and 4 °C. The supernatant was loaded onto a 1 ml glutathione Sepharose 4B column equilibrated with PBS. The resin was extensively washed with PBS, equilibrated with PreScission protease buffer (50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol), and incubated with 80 U PreScission protease (GE Healthcare) for 12 h at 4 °C. Each enzyme was eluted in 2 ml PreScission protease buffer, and then stored at −20 °C after adding an equal volume of 80 % glycerol. The protein concentrations were determined as previously described (Pace *et al.*, 1995). The purity of the samples was analysed by SDS-PAGE.

Gel-filtration chromatography. The molecular masses of the purified Fn0625 (54 μg) and Fn1220 (200 μg) proteins were 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. In this procedure, the standard curve was produced using molecular mass standards (Kit for Molecular Weights 12 000–200 000; Sigma). The enzyme elution was monitored at 280 nm.

Enzyme assay. The activity levels of the purified products were examined by measuring the rate of formation of pyruvate or H_2S (Yoshida *et al.*, 2003). The reaction mixture (100 μl) contained 40 mM potassium phosphate buffer (pH 7.6), 1 nmol PLP, 10.9 (for Fn0625) or 1.1 ng (for Fn1220) of the purified enzyme, and various concentrations of substrate(s). To determine pyruvate production, the reaction was terminated by adding 50 μl 4.5 % trichloroacetic acid after a 10 min incubation at 37 °C. The reaction mixture was centrifuged, and 250 μl of the supernatant was added to 750 μl of 0.33 M sodium acetate (pH 5.2) containing 0.017 % 3-methyl-2-benzothiazolinone hydrazone. The reaction mixture was then incubated at 50 °C for 30 min (Soda, 1968). The amount of pyruvate was determined by measuring A_{335}. Alternatively, to estimate H_2S production, a methylene blue formation assay was performed following the method of Schmidt (1987), with minor modifications. Briefly, the reaction (200 μl) was incubated at 37 °C for 10 min, and terminated by the addition of 20 μl of solution I (20 mM *N*′,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl) and 20 μl of solution II (30 mM $FeCl_3$ in 1.2 M HCl). After incubation for 30 min at room temperature, methylene blue formation was examined spectrophotometrically at 670 nm using a standard curve, which was made by measuring pyruvate in the reaction mixture containing L-cysteine and βC–S lyase from *Streptococcus anginosus*

Table 1. Oligonucleotide primers used in this study

Designation	Sequence (5′ to 3′)*
Purification of Fn0625	
021709-0625-GST-Bam	ATGGATCCGAAAAAGAAAAATTTTTAAAAGAATACTTAG
021709-0625-GST-Sal	AAGTCGACTTATCTATCACTCCTTAATTTTTTATATTCAC
Purification of Fn1220	
030209-FN1220-F-Bam	ATGGATCCTTAGCAAATTCTGTAATTGATTTAATTG
030209-FN1220-R-Sal	ATGTCGACTTCTTAAAACTTTTATAAATCACAAATATTAG
Real-time PCR	
042109-Fn0625-F	TCTATGTGGGTTGCAGATATGGAA
042109-Fn0625-R	TGCCCCATGTTCTATTCTTTCTTT
042109-Fn1220-F	TGCTTCATCTCCATTGCTTTCA
042109-Fn1220-R	ATCATAAACTGCTGGAATACCACCTA
081909-fn0654-Real-F	TGCTAAGACTGTTGTATGGAATGGA
081909-fn0654-Real-R	ACATACTCCTATTGTTCCTTTTGCAA
081909-fn2054-Real-F	CCCTGCTTCTGTTGACTTAACAAC
081909-fn2054-Real-R	AGGTTTCTTCTGCCATCTTGGA

*Underlining indicates restriction endonuclease sites incorporated to facilitate cloning.

(Ito *et al.*, 2008), since the enzyme produces equal amounts of pyruvate and H_2S from L-cysteine (Yoshida *et al.*, 2003).

The 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. To detect volatile sulfur compounds, including H_2S, methyl mercaptan, ethyl mercaptan, diethyl sulfide and dimethyl sulfide, gas chromatography was performed as described previously (Yoshimura *et al.*, 2000) with minor modifications. The reaction mixture (0.1 ml) contained 13.7 μg recombinant protein, 1 mM substrate, 10 μM PLP and 40 mM potassium phosphate buffer (pH 7.6). The reaction mixture was incubated in a 10 ml glass tube at 37 °C, followed by sealing with a silicone plug. After a 10 min incubation, a sample (1 ml) of the vapour above the reaction mixture in the tube was removed with a gas-tight syringe without taking out the 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 homocysteine, cysteine, cystathionine, *S*-hydroxyethylcysteine and 2-(2-aminoethylsulfanyl)ethanamine, which were predicted end products in the reaction mixtures, were investigated by reversed-phase HPLC. The reaction mixtures contained the following reagents in a final volume of 100 μl: 40 mM potassium phosphate buffer (pH 7.6), 10 μM PLP, 1 mM substrate(s), and 5.2 μg purified enzyme. After the mixtures had been incubated for 2 h at 37 °C, the enzymes were removed using a Microcon YM-10 filter (10 kDa cutoff; Amicon). The ultrafiltration products were dansylated as previously described (Tapuhi *et al.*, 1981). Aliquots (20 μl) of each sample were applied onto an XTerra RP_{18} column (4.6 × 150 mm; Waters) by injection. The dansylated products were separated at a flow rate of 1.0 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 of 350 and 530 nm, respectively, were used.

Liquid chromatography coupled with mass spectrometry (LC-MS). The fractions collected from HPLC were analysed using an LC-MS system consisting of an ion-trap mass spectrometer (HCT Ultrasil; Bruker Daltonics) equipped with a capillary LC (Agilent 1100 system) and Chemistation software (Agilent). The chromatographic analyses were performed with a reversed-phase Zorbax 300S-C_{18} column (150 mm × 0.3 mm; Agilent), and a gradient was applied of 15–50 % (v/v) acetonitrile/water containing 0.1 % (v/v) formic acid for 20 min at a flow rate of 4 μl min^{-1}. Analyte ionization was achieved by using positive electron spray ionization, a process that mainly produces the protonated molecular mass ion $(M+H)^+$. The temperature of the ESI capillary was maintained at 300 °C. The drying gas was introduced at a temperature of 300 °C and a flow rate of 4 l min^{-1}. The nebulizer gas pressure was 10 p.s.i. (69 kPa). The samples collected were evaporated to remove the solvent and then dissolved in 0.1 % formic acid in water. One microlitre of each sample was injected into the capillary LC.

Real-time quantitative PCR analysis. Overnight cultures of *F. nucleatum* ATCC 25586 were diluted (1/50) in fresh Columbia broth. The growth of cells incubated for 4, 6.5, 8.5, 12 and 24 h was monitored by determining the OD_{600}, and the cells collected at each incubation point were used for RNA extraction. Total RNA was isolated from the harvested cells using FastPrep Blue tubes (Bio 101). Contaminating DNA was eliminated by digestion with RNase-free DNase (Takara Bio). RNA (10 ng) was reverse transcribed into single-stranded cDNA using each reverse primer listed in Table 1. Real-time quantitative PCR amplification, detection and analysis were performed using the Thermal Cycler Dice RealTime System (Takara Bio) with SYBR *Premix Ex Taq* II (Takara Bio). Real-time PCR was carried out in 25 μl reaction mixtures (1 × SYBR *Premix Ex Taq* II, 22.5 pmol of each forward and reverse primers and 2.5 μl of template). The reaction conditions were 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. At the end of each run, a dissociation protocol (95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s) was performed to ensure that non-specific PCR products were absent. Primers (Table 1) were designed using the Primer Express software (version 3.0; Applied Biosystems). To estimate the initial amounts of

template in each sample, serial real-time PCR was performed using purified genomic DNA. For each gene, a standard curve was plotted using the log of the initial quantity of template against the threshold cycle (i.e. the cycle at which the fluorescence rose above the background level). In this way, differences in primer efficiency could be accommodated. Relative changes in gene expression were indicated as the difference (*n*-fold) relative to the values for calibrator cDNAs of two constitutive housekeeping genes: *fn0654*, encoding a phosphoglycerate kinase, and *fn2054*, encoding a glucose-6-phosphate isomerase (Qi *et al.*, 2005). The data reported were obtained from three independent experiments.

RESULTS

Multiple enzymes produce H_2S in *F. nucleatum*

The combination assay of SDS-PAGE and activity staining previously demonstrated that Cdl was the only enzyme to produce H_2S from L-cysteine in *F. nucleatum* subsp. *polymorphum* ATCC 10953 (Fukamachi *et al.*, 2002). Despite these findings, SDS-PAGE analysis with renaturation followed by activity staining, a method which was recently developed (Yoshida *et al.*, 2010), showed that crude enzyme extracts from the same strain contained multiple H_2S-producing enzymes, including three major proteins with non-uniform migration distances (Fig. 1a). Similar protein bands associated with H_2S production were also observed in crude enzyme extracts from *F. nucleatum* subsp. *nucleatum* ATCC 25586, although the protein with the longest migration distance in strain ATCC 10953 appeared to be slightly smaller than the corresponding protein in strain ATCC 25586.

Identification of Fn0625 and Fn1220 as H_2S-producing proteins in crude extracts of strain ATCC 25586

Database analysis showed that the open reading frame *fn1220* in the genomic DNA of *F. nucleatum* subsp. *nucleatum* ATCC 25586 (Kapatral *et al.*, 2002) is a homologue of the *cdl* gene in *F. nucleatum* subsp. *polymorphum* ATCC 10953. The amino acid sequence deduced from *fn1220* in strain ATCC 25586 was 97 % identical to the *cdl* gene product in strain ATCC 10953. Like the Cdl protein, Fn1220 showed no significant homology with βC–S lyase, which catalyses the α,β-elimination of L-cysteine to produce H_2S, pyruvate and ammonia. Instead, a database analysis demonstrated that the amino acid sequence of Fn0625 was 29–40 % identical with the βC–S lyase proteins encoded by *malY* in *E. coli* (Zdych *et al.*, 1995), *ytjE* in *Lactococcus lactis* (Martinez-Cuesta *et al.*, 2006; Sperandio *et al.*, 2005), *hly* in *Treponema denticola* (Chu *et al.*, 1995) and *lcd* in streptococcal species (Ito *et al.*, 2008; Yoshida *et al.*, 2003, 2008). To identify the Fn0625 and Fn1220 proteins in the crude enzyme extracts of strain ATCC 25586, the recombinant proteins were purified (Fig. 1a). The molecular masses of the denatured polypeptides were 47 and 33 kDa for the *fn0625* and *fn1220* products, respectively,

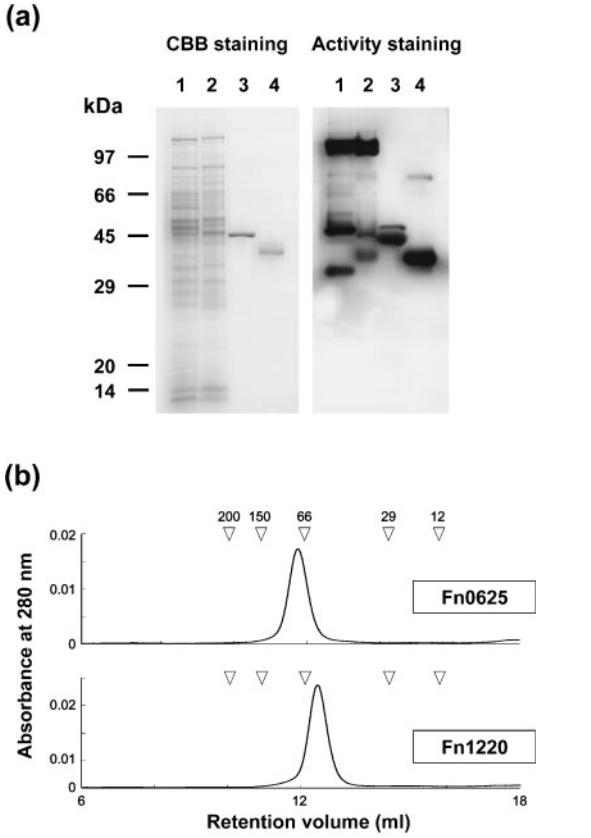

Fig. 1. Analyses of crude enzyme extracts and purified proteins of *F. nucleatum.* (a) SDS-PAGE analyses. Left panel: the gel was stained with Coomassie brilliant blue. Right panel: after the gel had been renatured, the enzymic activity was visualized by activity staining. Lanes: 1, cell lysate from *F. nucleatum* subsp. *polymorphum* ATCC 10953; 2, cell lysate from *F. nucleatum* subsp. *nucleatum* ATCC 25586; 3, purified Fn0625 of *F. nucleatum* subsp. *nucleatum* ATCC 25586; 4, purified Fn1220 of *F. nucleatum* subsp. *nucleatum* ATCC 25586. Five microlitres of each sample was applied to the gel. Positions of molecular mass markers are indicated. (b) Gel-filtration chromatography of purified Fn0625 (top) and Fn1220 (bottom) on Superdex 200 HR10/30. The elution volumes of molecular mass standards are shown as triangles with the protein size in kDa.

which agreed with the predicted molecular masses. In gel-filtration chromatography, the purified Fn0625 and Fn1220 were eluted at retention volumes corresponding to molecular masses of 84.0 and 65.4 kDa, respectively, calculated using a standard curve made with several standard proteins (Fig. 1b). These values were roughly twice the theoretical molecular masses, suggesting that both proteins are present as homodimers in solution. The SDS-PAGE analysis with renaturation followed by activity staining revealed that the two bands with long migration distances in crude enzyme extracts from strain ATCC 25586 corresponded to the *fn1220* and *fn0625* products (Fig. 1a). Identification of Cdl as the protein of lower molecular mass in the crude extracts of strain ATCC 10953

was also confirmed by comparing its electrophoretic mobility to recombinant purified Cdl (data not shown).

Enzymic characterization of purified Fn0625

To evaluate the enzymic activity of Fn0625, the breakdown of several substrates was determined by assaying for the production of ammonia, pyruvate and sulfur-containing compounds. Incubation of each substrate listed in Table 2 with purified Fn0625 consistently resulted in the production of both ammonia and pyruvate. The sulfur-containing compounds expected as end products in each reaction mixture were also detected using gas chromatography or HPLC with commercially available standards. Interestingly, cysteine, which was predicted to form by β-elimination of lanthionine, was not detected, probably because any cysteine produced would be rapidly degraded into ammonia, pyruvate and H_2S. Indeed, gas chromatography analysis confirmed that H_2S was produced in the reaction mixtures. Incubation of Fn0625 with *S*-(2-aminoethyl)-L-cysteine, L-methionine or L-homocysteine did not produce ammonia or pyruvate. These results suggested that the *fn0625* gene encodes a βC–S lyase that cleaves αC–N and βC–S linkages in substrates, but has no γC–S activity.

The kinetic properties for the decomposition of the substrates by Fn0625 were calculated from Lineweaver–Burk plots, and are summarized in Table 2. The k_{cat} and k_{cat}/K_m values of Fn0625 for L-cysteine were low compared to those for other substrates, suggesting that the capacity of Fn0625 to produce H_2S was low.

Fn1220 produces H_2S and lanthionine from L-cysteine

The kinetic values of Fn1220 for L-cysteine were also determined: K_m, 1.04 ± 0.07 mM; k_{cat}, 0.244 ± 0.01 s^{-1}; and k_{cat}/K_m, 0.237 ± 0.01 s^{-1} mM^{-1}. These values were obtained by measuring the amounts of H_2S produced using a methylene blue formation assay, since incubation of the purified Fn1220 with L-cysteine resulted in no production of pyruvate or ammonia. This unexpected finding led to the hypothesis that one or more byproducts, other than pyruvate and ammonia, might be produced with H_2S in the reaction mixture. HPLC analysis showed that the reaction of Fn1220 with L-cysteine yielded one product, which was dansylated before injection (Fig. 2a). This product was not observed in the reaction mixture incubated with L-cysteine and Fn0625. The other peak with the longest retention time, which was commonly observed, was unidentified.

To determine the mass of the unknown dansylated product using LC-MS, the peak was pooled from HPLC runs, evaporated to remove the solvent (i.e. methanol, acetic acid and triethylamine), and dissolved in distilled water. The molecular mass of the dansylated product was 442.10 in the MS spectrum, which corresponded to an $[M+H]^+$ ion of dansylated lanthionine (Fig. 2b). Further confirmation of the identity of the targeted metabolite was accomplished using HPLC with commercially available lanthionine, which was dansylated before injection (Fig. 2a).

Substrate specificity of Fn1220

To further understand the enzymic properties of Fn1220, its substrate specificity was investigated. Incubation of Fn1220 with several substrates that were degraded by Fn0625 mostly failed to produce ammonia or pyruvate (Table 3). However, these byproducts were produced when L-cystathionine was used as a substrate. The production of homocysteine, which is a sulfur-containing end product generated by β-elimination of L-cystathionine, was confirmed using HPLC. Thus, of all the tested substrates, only L-cystathionine was used as a substrate for the β-elimination reaction by Fn1220. The K_m and k_{cat} values

Table 2. Kinetic properties for the reactions catalysed by Fn0625 of *F. nucleatum* ATCC 25586

Substrate*	End product			K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
	Ammonia†	Pyruvate†	Sulfur-containing compound			
L-Cysteine	+	+	H_2S‡	0.22 ± 0.01	0.016 ± 0.001	0.073 ± 0.002
L-Cystine	+	+	H_2S	1.48 ± 0.20	24.31 ± 2.03	16.82 ± 1.31
S-Ethyl-L-cysteine	+	+	Ethyl mercaptan‡	41.56 ± 3.56	29.04 ± 3.33	0.70 ± 0.02
S-Methyl-L-cysteine	+	+	Methyl mercaptan‡	39.30 ± 5.20	15.06 ± 0.87	0.39 ± 0.03
L-Cystathionine	+	+	Homocysteine§	0.81 ± 0.06	27.75 ± 0.51	34.55 ± 1.79
DL-Lanthionine	+	+	Cysteine‖	3.49 ± 0.17	0.69 ± 0.01	0.20 ± 0.01

*No end products were detected from *S*-(2-aminoethyl)-L-cysteine, L-methionine or L-homocysteine.
†Plus sign indicates product detected.
‡Determined by gas chromatography.
§Determined by HPLC.
‖Instead of cysteine, which was a predicted end product in the reaction, H_2S was detected using gas chromatography.

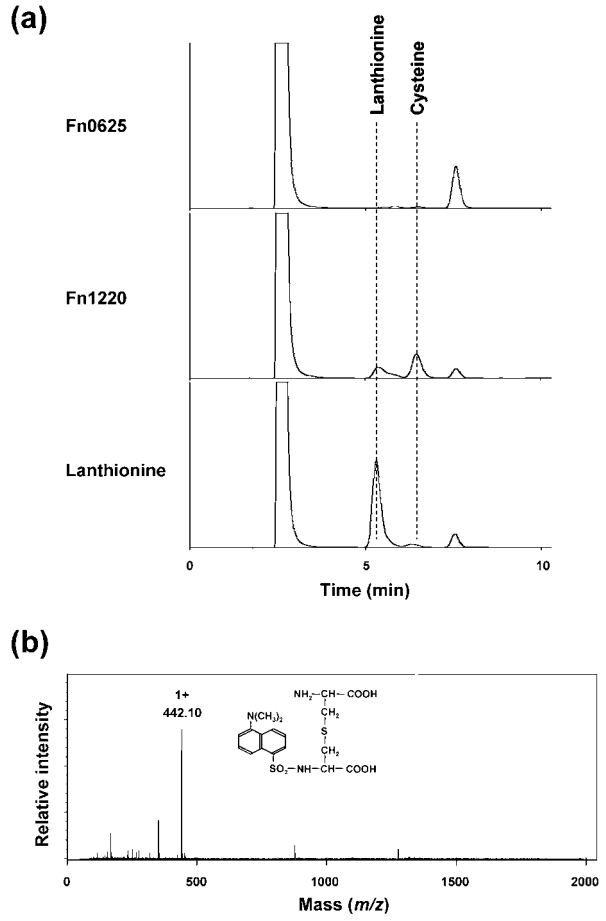

Fig. 2. Analyses of products in the reactions of L-cysteine with purified enzymes. (a) Reversed-phase HPLC profiles of dansylated reaction products. Top panel: reaction products obtained with purified Fn0625. Middle panel: reaction products obtained with purified Fn1220. Bottom panel: pure standard lanthionine. Peaks were identified by retention time. (b) Mass spectra of unidentified peak samples pooled from HPLC. The chemical structure of dansylated lanthionine is shown.

were 0.88 ± 0.04 mM and 0.39 ± 0.01 s^{-1}, respectively. In contrast, HPLC analysis showed that incubation of Fn1220 with L-cystathionine, *S*-(2-aminoethyl)-L-cysteine, *S*-methyl-L-cysteine or *S*-ethyl-L-cysteine (reactions 2–5 in Table 3) did not produce lanthionine, which is commonly produced by β-replacement of those substrates. Other sulfur-containing molecules predicted to form in reactions 3–5 were also not detected. Gas chromatography and HPLC analyses also failed to detect the predicted products in reactions 6–8 in Table 3, where two distinct substrates were used. The γ-replacement reactions of methionine and homocysteine were predicted to produce a pair of homolanthionine and dimethylsulfide, and a pair of homolanthionine and H_2S, respectively (reactions 9 and 10 in Table 3). Gas chromatography analysis indicated that dimethyl sulfide and H_2S were not produced in the reactions of Fn1220 with methionine or homocysteine.

Expression of *fn0625* and *fn1220* in *F. nucleatum* ATCC 25586

Real-time PCR analysis was carried out to evaluate the relative abundance of transcripts of the *fn0625* and *fn1220* genes in several cell growth phases of *F. nucleatum* (Fig. 3). The amounts of cDNA for these genes were normalized using the housekeeping gene *fn0654*, which encodes phosphoglycerate kinase. The cDNA levels of *fn0625* relative to *fn0654* ranged from 0.2 to 0.5 in any growth phase tested. The relative amount of *fn1220* to *fn0654* in cells incubated for 4 h was similarly low (0.1), whereas the values increased markedly in exponential-phase cells. For example, in the *F. nucleatum* cells grown for 8.5 h, *fn1220* was expressed 4.1- and 8.2-fold more than *fn0654* and *fn0625*, respectively. The results were similar when another housekeeping gene, *fn2054* (*pgi*), was used for normalization (data not shown).

DISCUSSION

F. nucleatum has been recognized as one of the most active oral bacteria in terms of H_2S production (Persson *et al.*, 1990). However, its mechanism of H_2S production has not been exactly understood, even though L-cysteine sulfhydrase was reported to be responsible for H_2S production in this species (Fukamachi *et al.*, 2002; Pianotti *et al.*, 1986). The current study revealed that H_2S production in *F. nucleatum* ATCC 25586 involves at least two enzymes: the previously identified Fn1220 and the newly identified Fn0625. Furthermore, this study found that Fn0625 functions as a βC–S lyase, and that Fn1220 catalyses the β-replacement reaction, which condenses two molecules of L-cysteine to generate H_2S, and as a side product, the uncommon amino acid lanthionine (Fig. 4).

The k_{cat}/K_m value of Fn0625 for L-cysteine (0.07 s^{-1} mM^{-1}) was approximately 29 % of the value for Fn1220 (0.24 s^{-1} mM^{-1}). Fn0625 also had a low value, compared with other bacterial βC–S lyases reported (Alting *et al.*, 1995; Chu *et al.*, 1997; Yoshida *et al.*, 2002, 2008). Taking this together with the relatively low levels of *fn0625* cDNA (Fig. 3), Fn0625 did not seem to contribute greatly to H_2S production in *F. nucleatum*. In contrast, Fn0625 catalysed α,β-elimination with a variety of substrates (Table 2). A wide substrate range is common for βC–S lyases. Reported βC–S lyases are mostly associated with the degradation of L-cystathionine to produce homocysteine, an intermediate indispensable for the methionine biosynthetic pathway. Of the calculated k_{cat}/K_m values of Fn0625, that for L-cystathionine was the highest (34.6 s^{-1} mM^{-1}), and this value was approximately 500 times greater than for L-cysteine. Thus, the reaction of Fn0625 with L-cysteine is probably coincidental to its main function. Fn0625 may be important for homocysteine production rather than for H_2S production.

Despite showing little identity with βC–S lyases from several micro-organisms, the amino acid sequence of

Table 3. Possible reactions catalysed by elimination or replacement of Fn1220 from *F. nucleatum* ATCC 25586

Reaction	Substrate(s)	β-Elimination reaction	β- or γ-replacement reaction			
		Catalysed by Fn1220*	Catalysed by Fn1220	Predicted end products		Replacement type
1	L-Cysteine	No	Yes	Lanthionine†	H_2S‡	β
2	L-Cystathionine	Yes	No	Lanthionine	Homolanthionine	β
3	*S*-(2-Aminoethyl)-L-cysteine	No	No	Lanthionine	2-(2-Aminoethylsulfanyl)-ethanamine†	β
4	*S*-Methyl-L-cysteine	No	No	Lanthionine	Dimethyl sulfide‡	β
5	*S*-Ethyl-L-cysteine	No	No	Lanthionine	Diethyl sulfide‡	β
6	L-Serine and homocysteine	ND	No	Cystathionine†		β
7	L-Cysteine and 2-mercaptoethanol	ND	No	*S*-Hydroxyethylcysteine†	H_2S	β
8	L-Cysteine and homocysteine	ND	No	Cystathionine	H_2S	β+γ
9	Methionine	No	No	Homolanthionine (ND)	Dimethyl sulfide	γ
10	Homocysteine	No	No	Homolanthionine (ND)	H_2S	γ

ND, Not determined.
*Determined by detecting ammonia and pyruvate.
†Determined by HPLC.
‡Determined by gas chromatography.

Fn1220 was 42 % and 40 % identical to cysteine synthases A and B in *E. coli* (Boronat *et al.*, 1984), respectively. Cysteine synthase, also known as *O*-acetylserine sulfhydrolase, produces L-cysteine and acetate from *O*-acetyl-L-serine and sulfide via β-replacement of the acetyl group with a thiol group (Becker *et al.*, 1969; Kredich & Tomkins, 1966). Recently, cysteine synthases were reported to produce H_2S in *E. coli*, although the production mechanism remains to be addressed (Awano *et al.*, 2005). Considering the identity of the amino acid sequences between Fn1220 and *E. coli* cysteine synthases, it is possible that H_2S production by *E. coli* cysteine synthases is associated with β-replacement of two molecules of L-cysteine, where lanthionine may be produced as a byproduct. Likewise, the Fn1220 protein may function as a cysteine synthase to produce L-cysteine and acetate from *O*-acetyl-L-serine and sulfide.

To our knowledge, only two enzymes in mammalian cells, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), have been reported so far to produce H_2S and lanthionine via the condensation of two molecules of L-cysteine (Chen *et al.*, 2004; Chiku *et al.*, 2009). Fn1220 is significantly identical (37 %) to human CBS, but not to CSE. Although the normal cellular function of CBS is to catalyse the condensation of serine with homocysteine to form cystathionine and water, this enzyme can efficiently produce H_2S via (i) a β,γ-replacement reaction in which cysteine is condensed with homocysteine to form H_2S and cystathionine; (ii) a β-elimination reaction in which cysteine is degraded to form H_2S and serine; and (iii) a β-replacement reaction in which two molecules of L-cysteine are condensed to generate H_2S and lanthionine (Chen *et al.*, 2004). Of these three reactions, the first has been estimated to be mainly responsible for H_2S production in human cells (Singh *et al.*, 2009). By contrast, CSE has a capacity to produce H_2S by five different reactions: (i) a β-elimination reaction in which cysteine is degraded to form H_2S, pyruvate and ammonia; (ii) a γ-elimination reaction in which homocysteine is degraded to form H_2S, α-ketobutyrate and ammonia; (iii) a γ-replacement reaction condensing two molecules of homocysteine to yield H_2S and homolanthionine; (iv) a β-replacement reaction condensing cysteine and homocysteine to yield H_2S and cystathionine; and (v) a β-replacement reaction condensing two molecules of cysteine to yield H_2S and lanthionine. These reactions were described in order of quantitative contribution to H_2S production by Singh *et al.* (2009). The β-replacement reaction that condenses two molecules of L-cysteine to generate H_2S and lanthionine by CBS and CSE – which are reactions (iii) and (v), respectively – is quantitatively less significant. In contrast, Fn1220 was not associated with the H_2S-producing reactions that CBS and CSE catalyse, except for the β-replacement of L-cysteine, suggesting that the substrate specificity of Fn1220 is limited (Table 3).

Peptidoglycans isolated from Gram-negative bacteria normally contain the amino acids D-glutamic acid, *meso*-diaminopimelic acid (A_2pm) and alanine. Interestingly, lanthionine, the analogue of A_2pm, was shown to be a natural constituent of the peptidoglycans isolated from *F. nucleatum* ATCC 25586, replacing A_2pm (Kato *et al.*, 1979).

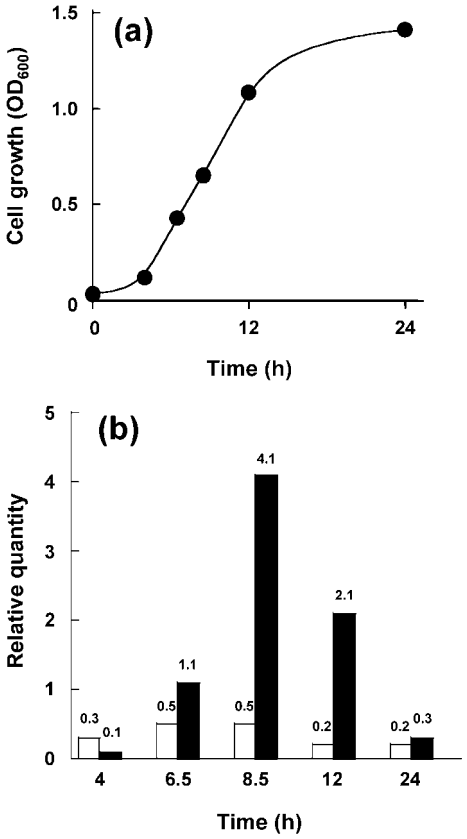

Fig. 3. Real-time quantitative PCR analysis of *fn0625* and *fn1220* expression in *F. nucleatum* subsp. *nucleatum* ATCC 25586. (a) Growth curve. The OD_{600} of bacterial samples used to obtain RNA was measured. Total RNAs were extracted from cells incubated for 4, 6.5, 8.5, 12 and 24 h. (b) Relative quantities of *fn0625* (white bars) and *fn1220* (black bars) transcripts during cell growth. The amount of each cDNA was indicated normalized against the amount of *fn0654* (a housekeeping gene encoding phosphoglycerate kinase) cDNA following reverse transcription of total RNA. The data are mean values from three experiments. The cDNAs used in each experiment were generated from independently isolated RNAs.

Based on these findings, Fn1220 might play important roles in the production of lanthionine. Interestingly, the uncommon amino acid lanthionine was found exclusively in the peptidoglycans of some species of *Fusobacterium*, including *F. nucleatum*, *F. necrophorum*, *F. russi* and *F. gonidiaformans* (Vasstrand *et al.*, 1982), for which homologue genes of *fn1220* may be ascribed a function as a taxonomic marker. Further studies are necessary to determine if this is the case.

Native PAGE with activity staining analysis by Claesson *et al.* (1990) demonstrated that multiple H_2S-producing enzymes, which showed large variations in electrophoretic mobility, were found not only in 12 species of *Fusobacterium*, but also in 6 strains of *F. nucleatum*. Using native PAGE and SDS-PAGE with activity staining analyses, *F. nucleatum* subsp. *polymorphum* ATCC 10953 was reported to contain only one

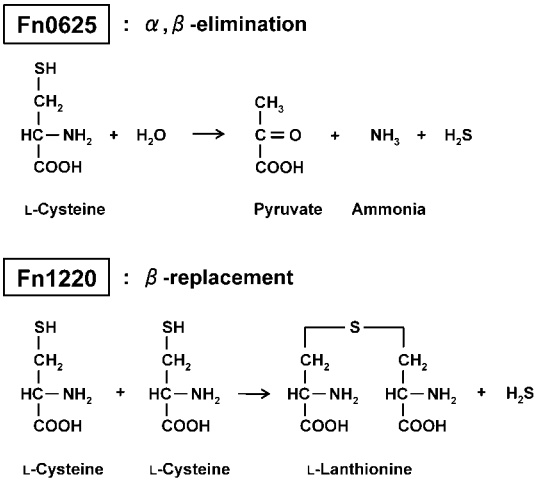

Fig. 4. Reactions catalysed by Fn0625 and Fn1220 to produce H_2S.

H_2S-producing enzyme, encoded by *cdl* (Fukamachi *et al.*, 2002). Our recently developed SDS-PAGE analysis with renaturation followed by activity staining (Yoshida *et al.*, 2010) demonstrated that strains ATCC 10953 and ATCC 25586 each contain at least three H_2S-producing enzymes, including Cdl (Fig. 1a). Such conflicting findings might be due to the enzymes possessing a quaternary structure when shifted to a higher molecular mass in the native gel or to inactivation of the enzymes in the SDS gel (Fukamachi *et al.*, 2002). Of the three major enzymes with H_2S-producing activities, two were identified in this study. However, the remaining one with the highest molecular mass (approx. 130 kDa), found in both ATCC 10953 and ATCC 25586, has not been identified. In *E. coli*, five different enzymes that produce H_2S have been identified (Awano *et al.*, 2005). As described above, several enzymes produce H_2S and they may have a wide range of different, but partly coinciding, substrate specificities. Further studies of the other enzymes associated with H_2S production in *F. nucleatum* are under way to characterize more precisely the mechanisms it uses to produce H_2S.

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