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* H₂S 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 H₂S-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 H₂S 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 H₂S 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 H₂S in distinct manners: Fn0625 carries out β-elimination of L-cysteine to produce H₂S, pyruvate and ammonia, whereas Fn1220 catalyses the β-replacement of L-cysteine to produce H₂S and lanthionine, the latter of which may be used for peptidoglycan formation in *F. nucleatum*.

INTRODUCTION

Hydrogen sulfide (H₂S) 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 H₂S-producing micro-organisms among tongue biofilm microflora (Washio et al., 2005). It has been demonstrated that, in vitro, H₂S damages epithelial cells (Morhart et al., 1970) and increases the permeability of oral mucosa (Ng & Tonzetich, 1984). More recently, it was reported that H₂S 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 H₂S are highly toxic to tissues and host cells, although most sufferers perceive halitosis primarily as a cosmetic problem. When considering the toxicity of H₂S, it is of interest that the amount of H₂S 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.
**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× YT 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 OD₅₅₀ of ~0.8. The cells were then harvested by centrifugation at 15 000 r.p.m. and 4 °C, resuspended in 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, and incubated for 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 gluthathione 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 H₂S (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 A₅₅₀. Alternatively, to estimate H₂S 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 FeCl₃ in 1.2 M HCl). After incubation for 30 min at room temperature, the 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*. The purity of the samples was analysed by SDS-PAGE.

**Preparation of crude enzyme extracts.** Each strain of *F. nucleatum* was grown to an OD₅₅₀ of ~0.8. The cells were then harvested by centrifugation at 15 000 r.p.m. and 4 °C, resuspended in 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, and incubated for 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 gluthathione 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.
The kinetic parameters were computed from the Lineweaver–Burk transformation of the Michaelis–Menten equation. The kinetic parameters were calculated using the $V_{\text{max}}$ 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$_2$S, 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 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 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 % dimethyl polysiloxane 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 dyesalted as previously described (Tapiwu et al., 1981). Aliquots (20 µl) of each sample were applied onto an XTerra RP18 column (4.6 × 150 mm; Waters) by injection. The dansylated products 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 (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-C18 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.
Multiple enzymes produce H$_2$S in *F. nucleatum*

The combination assay of SDS-PAGE and activity staining previously demonstrated that Cdl was the only enzyme to produce H$_2$S 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$_2$S-producing enzymes, including three major proteins with non-uniform migration distances (Fig. 1a). Similar protein bands associated with H$_2$S 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$_2$S-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 β-C-S lyase, which catalyses the α,β-elimination of L-cysteine to produce H$_2$S, pyruvate and ammonia. Instead, a database analysis demonstrated that the amino acid sequence of Fn0625 was 29–40% identical with the β-C-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, 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 \( \beta \)-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, 1-methionine or L-homocysteine did not produce ammonia or pyruvate. These results suggested that the \( fn0625 \) gene encodes a \( \beta \)C–S lyase that cleaves \( \beta \)C–N and \( \beta \)C–S linkages in substrates, but has no \( \gamma \)C–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: \( K_m = 1.04 \pm 0.07 \, \text{mM}; \, k_{cat} = 0.244 \pm 0.01 \, \text{s}^{-1}; \) and \( k_{cat}/K_m = 0.237 \pm 0.01 \, \text{s}^{-1} \, \text{mM}^{-1}. \) 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 \( \beta \)-elimination of L-cystathionine, was confirmed using HPLC. Thus, of all the tested substrates, only L-cystathionine was used as a substrate for the \( \beta \)-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**

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>End product</th>
<th>( K_m ) (mM)</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( k_{cat}/K_m ) (s(^{-1}) mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ammonia†</td>
<td>Pyruvate†</td>
<td>Sulfur-containing compound</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>+</td>
<td>+</td>
<td>( H_2S )‡</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>+</td>
<td>+</td>
<td>( H_2S )‡</td>
<td>1.48 ± 0.20</td>
</tr>
<tr>
<td>S-Ethyl-L-cysteine</td>
<td>+</td>
<td>+</td>
<td>Ethyl mercaptan‡</td>
<td>41.56 ± 3.56</td>
</tr>
<tr>
<td>S-Methyl-L-cysteine</td>
<td>+</td>
<td>+</td>
<td>Methyl mercaptan‡</td>
<td>39.30 ± 5.20</td>
</tr>
<tr>
<td>L-Cystathionine</td>
<td>+</td>
<td>+</td>
<td>Homocysteine§</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>DL-Lanthionine</td>
<td>+</td>
<td>+</td>
<td>Cysteine</td>
<td></td>
</tr>
</tbody>
</table>

*No end products were detected from S-(2-aminoethyl)-L-cysteine, 1-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.
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 H2S production (Persson et al., 1990). However, its mechanism of H2S production has not been exactly understood, even though l-cysteine sulfhydrylase was reported to be responsible for H2S production in this species (Fukamachi et al., 2002; Pianotti et al., 1986). The current study revealed that H2S 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 H2S, 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 H2S 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 H2S production.

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

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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.

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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 H2S, respectively (reactions 9 and 10 in Table 3). Gas chromatography analysis indicated that dimethyl sulfide and H2S were not produced in the reactions of Fn1220 with methionine or homocysteine.
Recently, cysteine synthases were reported to produce H₂S and B in 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 sulphydrolase, 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 H₂S 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 H₂S 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 H₂S 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 H₂S via (i) a β-γ-replacement reaction in which cysteine is condensed with homocysteine to form H₂S and cystathionine; (ii) a β-elimination reaction in which cysteine is degraded to form H₂S and serine; and (iii) a β-replacement reaction in which two molecules of L-cysteine are condensed to generate H₂S and lanthionine (Chen et al., 2004). Of these three reactions, the first has been estimated to be mainly responsible for H₂S production in human cells (Singh et al., 2009). By contrast, CSE has a capacity to produce H₂S by five different reactions: (i) a β-elimination reaction in which cysteine is degraded to form H₂S, pyruvate and ammonia; (ii) a γ-elimination reaction in which homocysteine is degraded to form H₂S, α-ketobutyrate and ammonia; (iii) a γ-replacement reaction condensing two molecules of homocysteine to yield H₂S and homolanthionine; (iv) a β-replacement reaction condensing cysteine and homocysteine to yield H₂S and cystathionine; and (v) a β-replacement reaction condensing two molecules of cysteine to yield H₂S and lanthionine. These reactions were described in order of quantitative contribution to H₂S production by Singh et al. (2009). The β-replacement reaction that condenses two molecules of L-cysteine to generate H₂S 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 H₂S-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 (A₂pm) and alanine. Interestingly, lanthionine, the analogue of A₂pm, was shown to be a natural constituent of the peptidoglycans isolated from F. nucleatum ATCC 25586, replacing A₂pm (Kato et al., 1979).

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

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Substrate(s)</th>
<th>β-Elimination reaction</th>
<th>β- or γ-replacement reaction</th>
<th>Predicted end products</th>
<th>Replacement type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Catalysed by Fn1220*</td>
<td>Catalysed by Fn1220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>L-Cysteine</td>
<td>No</td>
<td>Yes</td>
<td>Lanthionine†</td>
<td>H₂S‡</td>
</tr>
<tr>
<td>2</td>
<td>L-Cystathionine</td>
<td>Yes</td>
<td>No</td>
<td>Lanthionine</td>
<td>Homolanthionine</td>
</tr>
<tr>
<td>3</td>
<td>S-(2-Aminoethyl)-L-cysteine</td>
<td>No</td>
<td>No</td>
<td>Lanthionine</td>
<td>2-(2-Aminoethylysulfonyl)-ethanamine†</td>
</tr>
<tr>
<td>4</td>
<td>S-Methyl-L-cysteine</td>
<td>No</td>
<td>No</td>
<td>Lanthionine</td>
<td>Dimethyl sulfide‡</td>
</tr>
<tr>
<td>5</td>
<td>S-Ethyl-L-cysteine</td>
<td>No</td>
<td>No</td>
<td>Lanthionine</td>
<td>Diethyl sulfide‡</td>
</tr>
<tr>
<td>6</td>
<td>L-Serine and homocysteine</td>
<td>ND</td>
<td>No</td>
<td>Cystathionine†</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>L-Cysteine and 2-mercaptoethanol</td>
<td>ND</td>
<td>No</td>
<td>S-Hydroxyethylcysteine†</td>
<td>H₂S</td>
</tr>
<tr>
<td>8</td>
<td>L-Cysteine and homocysteine</td>
<td>ND</td>
<td>No</td>
<td>Cystathionine</td>
<td>H₂S</td>
</tr>
<tr>
<td>9</td>
<td>Methionine</td>
<td>No</td>
<td>No</td>
<td>Homolanthionine (ND)</td>
<td>Dimethyl sulfide</td>
</tr>
<tr>
<td>10</td>
<td>Homocysteine</td>
<td>No</td>
<td>No</td>
<td>Homolanthionine (ND)</td>
<td>H₂S</td>
</tr>
</tbody>
</table>

ND: Not determined.

* Determined by detecting ammonia and pyruvate.
† Determined by HPLC.
‡ Determined by gas chromatography.
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 *H₂S*-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 *H₂S*-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 *H₂S*-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 *H₂S*-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 *H₂S* have been identified (Awano et al., 2005). As described above, several enzymes produce *H₂S* and they may have a wide range of different, but partly coinciding, substrate specificities. Further studies of the other enzymes associated with *H₂S* production in *F. nucleatum* are under way to characterize more precisely the mechanisms it uses to produce *H₂S*.

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