3-Chloro-DL-alanine resistance by L-methionine-α-deamino-γ-mercaptomethane-lyase activity

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Abstract The antibacterial agent 3-chloro-DL-alanine (3CA) is an inhibitor of peptidoglycan synthesis. *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, the bacteria responsible for oral malodor, are shown to be resistant to 1 mM 3CA, whereas *Streptococcus mutans* and *Escherichia coli* are sensitive to this antibacterial agent at the same concentration. We isolated the 3CA resistance gene from *F. nucleatum* and showed that the gene encodes an L-methionine-α-deamino-γ-mercaptomethane-lyase that catalyzes the α,γ-elimination of L-methionine to produce methyl mercaptan. The enzyme also exhibits 3CA chloride-lyase (deaminating) activity. This antibacterial agent is expected to be useful for specific selection of malodorous oral bacteria producing high amounts of methyl mercaptan.

Key words: 3-Chloro-DL-alanine; L-Methionine; Methyl mercaptan; L-Methionine-α-deamino-γ-mercaptomethane-lyase; *Fusobacterium nucleatum*

1. Introduction

L-Methionine-α-deamino-γ-mercaptomethane-lyase (METase) catalyzes the transformation of L-methionine into methyl mercaptan, ammonia, and α-ketobutyrate. This enzyme catalyzes the α,β- or α,γ-elimination reaction of not only L-methionine but also L-homocysteine, L-cysteine, and *S*-adenosylmethionine. Methyl mercaptan, one of the products of METase, is a major cause of the pathological oral malodor associated with periodontitis [1]. Previously, we tested the ability of several bacteria to form methyl mercaptan from L-methionine. We found that *Fusobacterium nucleatum*, a Gram-negative, anaerobic, rod-shaped bacterium that is frequently recovered from infections in humans, produces large amounts of methyl mercaptan [2].

In seeking specific substrates for METase activity, we investigated 3-chloro-DL-alanine (3CA), which is also known to be an effective antibacterial agent. We tested the antibacterial activity of 3CA against several oral bacteria. The presence of 1 mM 3CA had no effect on the growth of *Porphyromonas gingivalis* and *F. nucleatum*, which produce large amounts of methyl mercaptan. In contrast, *Streptococcus mutans* and *Escherichia coli*, which do not produce methyl mercaptan from L-methionine [3], could not grow in the presence of 1 mM 3CA. 3CA also inhibited the growth of a METase-deficient mutant of *P. gingivalis*. Based on these results, we hypothesized that METase might play an important role in detoxifying 3CA. Thus, we turned our attention to the relationship between METase activity and 3CA resistance. In this report, we describe the cloning of the 3CA resistance gene and its product has been characterized.

2. Materials and methods

2.1. Chemicals

3CA was purchased from ICN Biomedicals. L-Methionine, L-cysteine, DL-homocysteine, L-ethionine and *S*-methyl-L-cysteine were purchased form Wako Pure Chemicals Industries. All other chemicals were reagent grade.

2.2. Enzyme analysis

Since METase degrades homocysteine to produce hydrogen sulfide [4–7], DL-homocysteine was used as a substrate for detection of METase activity. Enzyme reactions resulting in hydrogen sulfide production were assayed as described by Claesson et al. [8]. Proteins were suspended in a solution containing 100 mM triethanolamine-HCl (pH 7.6), 10 μM pyridoxal 5′-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, and 20 mM DL-homocysteine at 37°C. The bismuth in this solution reacts with sulfide forming a black precipitate. The hydrogen sulfide producing activity was also visualized by a modified method according to the procedure of Claesson et al. [8]. In this method, after electrophoresis of the enzyme, the gel is soaked at 37°C in a solution containing 100 mM triethanolamine-HCl (pH 7.6), 10 μM pyridoxal 5′-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100, and 20 mM homocysteine. A black precipitate forms at the site of the actual enzymes in the gel. The substrate specificity of purified METase was determined by assaying the production of α-ketobutyrate or pyruvate as described previously [2].

2.3. Purification of METase from F. nucleatum

F. nucleatum ATCC 10953 was grown anaerobically (10% CO_2, 10% H_2, 80% N_2) in GAM broth (Nissui Medical Co.) at 3°C for 20 h and harvested by centrifugation. The bacterial pellet (8.4 g) was suspended in 50 mM Tris–HCl (pH 7.5) containing 10 mM EDTA and 10 mM phenylmethylsulfonyl fluoride, and cells were lysed by ultrasonication. The suspension was centrifuged, and the supernatant was fractionated with ammonium sulfate. Proteins precipitating between 50 and 70% saturation with ammonium sulfate were collected by centrifugation. The precipitate was dissolved in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM EDTA and 10 mM pyridoxal 5′-phosphate and loaded on a 2.5×40-cm gel filtration chromatography column (TSK gel Toyopearl HW-55(F)) equilibrated with the same buffer. The protein was eluted at 0.25 ml/min with the same buffer, and the fraction corresponding to the peak of activity was desalted

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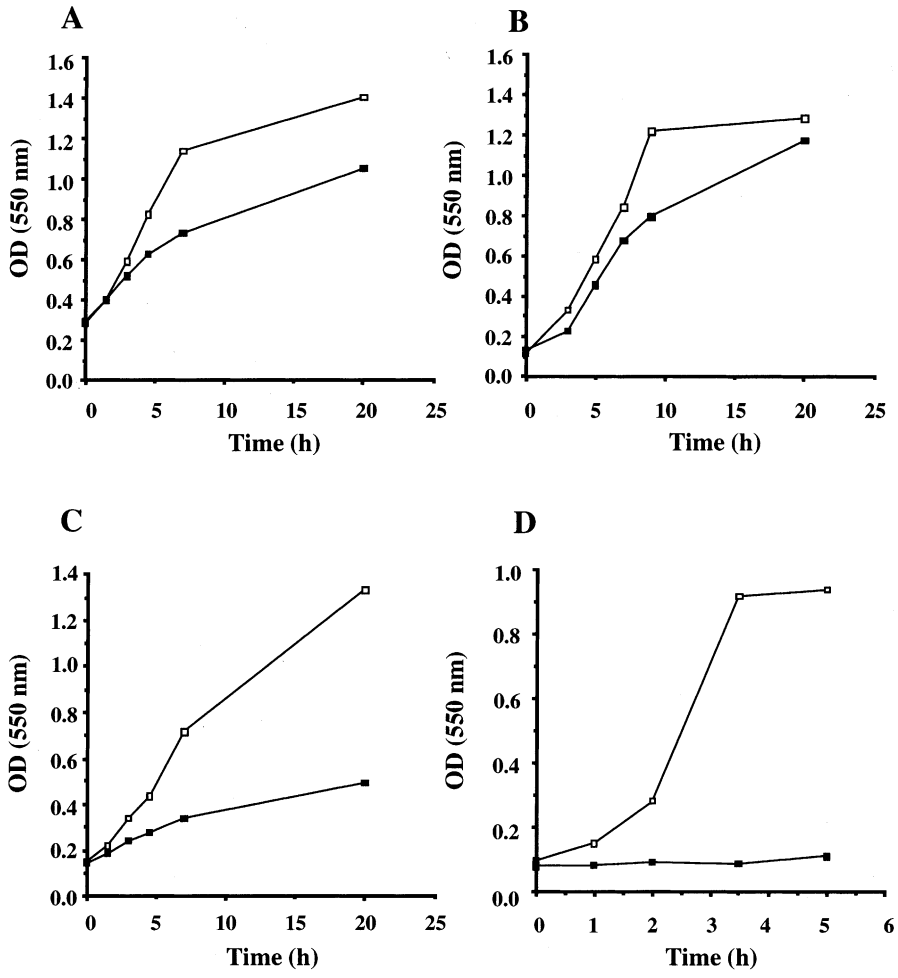

Fig. 1. Inhibition of bacterial growth by 3CA. *P. gingivalis* W83 (A), *F. nucleatum* ATCC 10953 (B), *P. gingivalis* M1217 (C), and *S. mutans* Xc (D) were grown in the presence (filled square) or absence (open square) of 1 mM 3CA.

and concentrated with a 30-kDa cutoff centrifugal filter unit (Millipore Corporation). The concentrated fraction was loaded onto an anion exchange column (TSK gel DEAE-5PW) equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The enzyme was eluted at 1 ml/min with a 40-ml linear gradient from 0.1 to 0.4 M NaCl. The fraction containing the activity was concentrated, and ammonium sulfate was added to a final concentration of 1.5 M. The sample was then purified by hydrophobic HPLC on a phenyl 5PW column (7.5×75 mm) equilibrated with 1.5 M ammonium sulfate dissolved in 0.1 M sodium phosphate buffer (pH 6.8). Proteins were eluted at 1 ml/min with a 40 ml descending gradient from 1.5 to 0 M ammonium sulfate. Proteins elution was monitored by measuring A_{280}. The peak with activity was pooled and desalted with a 30-kDa cutoff centrifugal fraction unit. All extraction steps were performed at 0 to 4°C.

2.4. Cloning of the F. nucleatum METase gene

To construct a cosmid gene library, chromosomal DNA from *F. nucleatum* ATCC 10953 was partially digested with *Apo*I, and fragments of DNA ranging from 5 to 15 kb in size were collected. The cosmid vector charomid 9-36 (Nippon Gene, Tokyo, Japan) was digested with *Eco*RI. Equimolar amounts of the vector and insert fragments were mixed and ligated, packaged into bacteriophage λ (Gigapack III XL; Stratagene), and transfected into *E. coli* DH5α cells. Positive subclones were selected based on growth on 2×TY plates supplemented with 3CA (1 mM) and ampicillin (50 μg/ml). Six colonies resistant to 3CA and ampicillin were chosen at random and tested for their activity to form hydrogen sulfide from DL-homocysteine. A plasmid isolated from the single positive colony was designated pFMC808 and used in further studies. The 6-kb *Eco*RV fragment of this plasmid that was essential for the ability to produce hydrogen sulfide was subcloned into pMCL210 [9] and the resultant plasmid was designated pFMC12. The nucleotide sequence of the insert in pFMC12 was determined by the dideoxy termination technique of Sanger et al. [10], using a Thermo Sequence II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) and an ABI 373A DNA sequencer (Applied Biosystems).

2.5. Purification of the recombinant F. nucleatum METase

To obtain the product of the recombinant gene, *E. coli* BL21 cells were transformed with a plasmid containing the *mgl* gene amplified by PCR using the primers 5′-CCCCCGGATCCGAAACGAAAAAA-TATGGT-3′ (sense) and 5′-CCCCCGTCGACTTAAATTTTTTC-TAGTCC-3′ (antisense). These primers were designed to create *Bam*HI and *Sal*I restriction sites (underlined) in the PCR product. The PCR product was double-digested with *Bam*HI and *Sal*I, and ligated to the *Bam*HI–*Sal*I double-digested pGEX-6P-1 Expression Vector (Pharmacia Biotech) to produce pFMGL110. The transformant was grown in 2×TY broth with ampicillin (50 μg/ml) at 37°C until an OD_{550} of 0.7 was attained. Isopropyl-β-thiogalactopyranoside was added to the culture at a final concentration of 1 mM, and the culture was grown for 4 h. The cells were harvested by centrifugation and lysed by ultrasonication. The cell extract was obtained by centrifugation. Binding to glutathione Sepharose 4B medium (Amersham Pharmacia Biotech), cleavage of the fusion protein by PreScission Protease, and elution of the product were performed according to the manufacturers' instructions.

2.6. Inhibition of bacterial growth by 3CA

Growth inhibition studies were conducted in 3-ml cultures inoculated with bacterial cells to an OD_{550} of ∼0.1, and maintained at

37°C. 3CA was added to a final concentration of 1 mM. Growth was monitored at OD_{550} until the control cultures reached the stationary phase.

3. Results and discussion

Preliminary experiments indicated that *P. gingivalis* strain W83 and *F. nucleatum* strain ATCC 10953 could grow in medium containing 1 mM 3CA (Fig. 1A,B), whereas the growth of *P. gingivalis* strain M1217, a METase-deficient mutant of W83 [2], was strongly inhibited (Fig. 1C). In contrast, *S. mutans* strain Xc could not grow in the presence of 1 mM 3CA (Fig. 1D). The growth of *E. coli* strain DH5α was similarly inhibited by 1 mM 3CA (data not shown). Although it has not been reported that METase is involved in the detoxification of 3CA, this result suggested that METase might play an important role in the resistance of *F. nucleatum* to 3CA. Thus, we hypothesized that *E. coli* would grow in the presence of 3CA when transformed by a plasmid containing the *F. nucleatum* METase gene. *E. coli* cells containing *F. nucleatum* library subclones were selected based on growth on 2×TY plates supplemented with 3CA (1 mM) and ampicillin (50 μg/ml). One of the colonies resistant to 3CA was able to produce hydrogen sulfide from homocysteine. From this colony, the 6-kb *Eco*RV fragment essential for the ability to produce hydrogen sulfide from DL-homocysteine was isolated and subcloned, and the resultant plasmid was designated pFMC12.

The nucleotide sequence of the insert in pFMC12 was determined. An 1188-bp open reading frame (ORF) was found within the insert, and the deduced amino acid sequence of this ORF predicted a 396-amino acid polypeptide with a molecular mass of 43.3 kDa. This polypeptide is highly similar in amino acid sequence to the METases of *P. gingivalis* [2], *Pseudomonas putida* [4,5] and *Trichomonas vaginalis* [7]. The amino acid sequence is 56.3% identical to the *P. gingivalis mgl* gene product, and 39.6 and 37.8% identical to the *P. putida mgl*1 and *mgl*2 gene products, respectively, and 49.2 and 44.9% identical to the *T. vaginalis mgl*1 and *mgl*2 gene products, respectively.

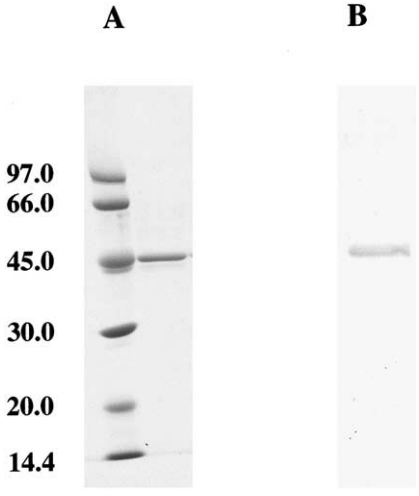

Fig. 2. SDS–PAGE analysis of purified METase from *F. nucleatum* ATCC 10953. The purified METase was mixed with SDS–PAGE loading buffer and heated at 100°C for 3 min. A sample (1.0 μg) was then subjected to SDS–PAGE (10% polyacrylamide). The positions of molecular size markers are indicated to the left. A: The gel was stained with Coomassie brilliant blue R-250. B: The gel was incubated at 37°C in solution containing 100 mM triethanolamine-HCl (pH 7.6), 10 μM pyridoxal 5′-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100, and 20 mM homocysteine to visualize the homocysteine desulfhydrase activity. A black precipitate was formed at the site of the METase enzyme in the gel.

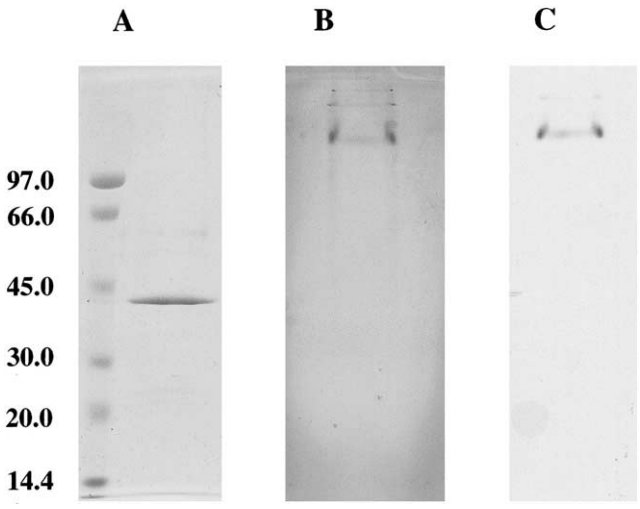

Fig. 3. PAGE analysis of recombinant METase. The *mgl* gene of *F. nucleatum* was expressed in *E. coli* and recombinant protein was purified by affinity chromatography. A: The sample was analyzed by SDS–PAGE and the gel was stained with Coomassie brilliant blue R-250. B: The sample was analyzed by native PAGE and the gel was stained with Coomassie brilliant blue R-250. C: The sample was analyzed by native PAGE and the homocysteine desulfhydrase activity was visualized as described above (Fig. 2B).

To confirm that the cloned gene encoded METase, the enzyme was purified from *F. nucleatum* ATCC 10953. Since METase is known to degrade homocysteine to produce hydrogen sulfide, DL-homocysteine was used as a substrate to detect METase activity. After the purified protein was subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), gels were either stained with Coomassie brilliant blue R-250 (Fig. 2A) or transferred to a polyvinylidene difluoride membrane. The prominent 43.3-kDa protein band was excised for N-terminal sequencing directly on the membrane by Edman degradation. The resulting N-terminal amino acid sequence (METKKYGLGTTAIHAGTLKN) was in agreement with the deduced amino acid of the ORF responsible for the ability to produce hydrogen sulfide from DL-homocysteine. The homocysteine desulfhydrase activity of the protein was also visualized directly in the gel after non-denaturing electrophoresis (Fig. 2B). The protein was found to produce methyl mercaptan and α-ketobutyrate from L-methionine (data not shown). These results demonstrate that the product of the cloned gene from *F. nucleatum* is a METase. The gene has been designated *mgl*, and the nucleotide sequence of the *mgl* gene of *F. nucleatum* has been assigned DDBJ accession number AB077041.

To evaluate the enzymatic activity of *F. nucleatum* METase, *E. coli* BL21 cells were transformed with pFMGL110 containing the *mgl* gene. The purified recombinant protein was subjected to SDS–PAGE and visualized with Coomassie brilliant blue R-250 (Fig. 3A). PAGE under non-denaturing conditions (native PAGE) was also carried out and the homocysteine desulfhydrase activity was visualized in the gel. The band

Table 1
Substrate specificities of recombinant METase of *F. nucleatum* ATCC 10953

Substrate	Relative activity (%)[a]
L-Methionine	100
DL-Homocysteine	170
3-Chloro-DL-alanine	247[b]
S-Methyl-L-cysteine	49[b]
L-Cysteine	3.6[b]
L-Ethionine	76

[a]Determined by α-ketobutyrate production.
[b]Determined by pyruvate production.

stained with Coomassie brilliant blue corresponded to the black precipitate (Fig. 3B,C). The substrate specificity of purified METase was determined by assaying the production of α-ketobutyrate or pyruvate as described previously [2]. 3CA was a better substrate by 2.5-fold than was L-methionine. L-Cysteine and *S*-methyl-L-cysteine were poor substrates (Table 1).

Methyl mercaptan produced from L-methionine by the enzymatic action of METase is considered to be one of the main causes of oral malodor associated with periodontitis. Yaegaki et al. reported that the ratio of methyl mercaptan to hydrogen sulfide was greater in mouth air from patients with periodontal disease than in air from control subjects [11]. Coil et al. found a significantly higher methyl mercaptan/hydrogen sulfide ratio in deep or inflamed crevicular sites than in shallow or non-inflamed sites [12]. These findings suggest that methyl mercaptan from periodontal pockets may be associated with the oral malodor of patients with periodontitis. In addition, Ng et al. reported that exposure of the sublingual porcine mucosa to methyl mercaptan increases the permeability of the tissue [13]. Methyl mercaptan has also been shown to induce interleukin-1β secretion from mononuclear cells [14]. Understanding the mechanism of methyl mercaptan production in periodontal pockets may not only help us elucidate the source of oral malodor, but may also provide a clue to understanding the pathology of periodontal disease.

The relationship between METase activity and 3CA chloride-lyase activity has not been previously reported. We have now shown that oral bacteria producing high amounts of methyl mercaptan are resistant to 3CA and that this resistance depends on METase activity. Thus, this antibacterial agent is expected to be useful for specifically selecting malodorous bacteria producing large amounts of methyl mercaptan from among the oral bacteria.

METase has been reported to inhibit the growth of malignant cells that have an absolute requirement for L-methionine by methionine deprivation. Thus, METase is thought to be useful as a tumor-selective therapeutic for effective, low-toxicity human cancer therapy. For effective enzymatic degradation of L-methionine, METase with a lower Michaelis constant have been sought in various bacterial species. 3CA may be useful for cloning of the METase genes from bacteria which contain high METase activity.

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